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THE IMMUNE-ADHERENCE ACTIVITY OF NORMAL SERUM

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A HEAT labile non-specific complement-fixing activity in normal serum was described by Mackie and Finkelstein (1930). A similar activity was described by Pillemer *et al.* (1954) which they called the properdin system. That part of the properdin system which is absorbed by zymosan and a wide range of polysaccharides has been shown to account for some of the bactericidal and viricidal activity of normal serum (Pillemer *et al.*, 1954 and 1955). Properdin appears to need Mg^{++} ions and serum co-factors resembling the 4 components of complement for its action.

The immune-adherence phenomenon (Nelson, 1953), in which an antigen coated with its specific antibody and after fixing all 4 components of complement will adhere to the primate red cell, provides a sensitive test with which to re-explore the immunological activity of normal sera. Antibody to particulate antigens can be titrated by counting the numbers of red cells with adherent antigens, in immune-adherence reaction mixtures, under the phase contrast microscope.

Nelson and Lebrun (1956) found that normal guinea-pig serum caused the immune-adherence of starch grains and Nelson and Kelsey (personal communication) found the same true for zymosan.

The present work describes the immune-adherence activity of normal sera with respect to certain particulate antigens. Two forms of activity are distinguished, one specific and the other non-specific. The relation between the non-specific immune-adhering activity and the properdin system are discussed.

MATERIALS AND METHODS

Veronal buffered saline with added Ca^{++} and Mg^{++} was used throughout as diluent (Fulton and Dumbell, 1949).

Antigens

Amaranthus cruentus starch (Nelson and Lebrun, 1956) was used as an antigen in immune-adherence experiments, because its size in relation to that of red cells enables grains adhering

to red corpuscles to be easily counted. Rice starch (B.D.H.) was used for absorbing sera. Zymosan was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio.

Bacteria were grown on nutrient agar, killed at 60° for 1-hr. and washed 3 times in saline before use.

Polysaccharides

Amylose, dextran and laminarin were kindly given by Dr. W. Whelan of the Lister Institute, London, yeast mannan by Dr. D. H. Northcote of the Department of Biochemistry, University of Cambridge, and levan by Dr. M. Shilo of the Department of Microbiological Chemistry, the Hebrew University, Jerusalem. Inulin was obtained from George T. Gurr Ltd., and xylan from the Nutritional Biochemical Corporation.

Sera

All sera were stored at −70°. All guinea-pig serum was pooled, each pool containing the sera from 6–8 animals. Samples of serum taken from newborn calves before suckling were kindly given by Dr. A. E. Pierce of the Agricultural Research Council's Animal Physiology Research Establishment, Babraham.

Complement

Undiluted guinea-pig serum for complement was absorbed twice at 4° with 50–100 mg. (dry weight) per ml. of the antigen to be used, and used in a dilution of 1/32.

Horse serum was used as a source of complement in experiments on the immune-adherence activity of pre-suckling calf serum and was similarly absorbed before use.

Erythrocytes

Human group O blood was stored in Alsever's solution at 4°. The cells were washed three times in veronal buffered saline before use.

Immune-adherence titrations

Antigen in 0·5 ml. amounts (2×10^8 bacteria/ml., or 0·125 mg./ml. of starch or zymosan), 0·2 ml. of the serum to be tested, titrated out in doubling dilutions, and 0·2 ml. of absorbed serum to provide an excess of complement, were incubated in Kahn tubes at 37° for 20 min. in a water bath. Volumes of 0·1 ml. of 1·5 per cent washed group O human red cells were added and the tubes replaced in the water bath 1 hr. The end point of the titration was judged roughly by the haemagglutination patterns which formed at the bottom of the tubes after this time. The cellular deposit at the bottom of the tubes was then examined under the phase contrast microscope and the titre was taken as the reciprocal of the initial dilution of serum in the last tube that contained 20 per cent or more red cells with adherent antigenic particles.

Bactericidal titrations

These were carried out in M.R.C. plastic haemagglutination trays using the technique of Nagington (1956).

Absorption of sera

Sera were absorbed twice at 4° with 50–100 mg. of bacteria (wet weight) per ml., or 100 mg./ml. of zymosan or polysaccharide, in 50 ml. flat-bottomed flasks on a horizontal rocking machine. All bacteria or water insoluble polysaccharides were washed 3 times in saline before being used for absorption.

Fractionation of serum

Sera were fractionated into euglobulin and pseudoglobulin fractions by dilution. The serum was diluted 1/10 in 0·005 M KH_2PO_4 at 0° for 30 min. The resulting precipitate was centrifuged off at 4° and washed 3 times in a phosphate solution pH 5·4 and ionic strength 0·02. It was redissolved in veronal buffered saline. The supernatant was brought back to isotonicity by the addition of 10 per cent NaCl, and the pH was brought back to 7·4 by the addition of 0·1 N NaOH.

RESULTS

The immune-adherence activity of normal guinea-pig, rabbit and human sera

Table I shows the titres of immune-adherence activity, in the presence of an excess of complement, of normal guinea-pig serum (derived from a pool of 8 guinea-pigs weighing between 350 and 400 g.), normal rabbit serum (adult 12-month-old rabbit weighing 3·5 kg.), and an adult male human (aged 27 yr.).

TABLE I.—*Titre* (1 :) *of Immune-adherence Activity of Normal Guinea-pig, Rabbit and Human Serum, in the Fresh Serum, Serum Heated at* 55° *for* 20 *min. and After Absorption with* 0·1 *g./ml. Zymosan twice at* 4°

	Guinea-pig (Pool of 8)			Rabbit (Adult male 3 kg. —1 yr old)			Human (Adult male age 27 yr.)		
	Fresh	Heated 55°	Zymosan absorbed	Fresh	Heated 55°	Zymosan absorbed	Fresh	Heated 55°	Zymosan absorbed
Salm. typhi	256	16	16	32	<8	<8	32	<8	16
Sh. flexneri	256	<8	16	128	128	128	128	8	64
Bact. coli	128	<8	16	128	8	8	128	<8	64
V. el tor	64	8	16	32	<8	<8	64	8	32
Staph. aureus	512	<8	16	128	16	8	64	8	32
Ery. rhusiopathiae	1024	1024	1024	32	<8	<8	32	<8	8
Starch	512	<8	<8	1024	1024	1024	64	<8	32

On absorbing the serum with 100 mg./ml. zymosan, or heating the serum at 55°, there was a marked fall in the activity of the guinea-pig serum against all antigens tested except *Erysipelothrix rhusiopathiae*, the titre against which was unaffected by both these processes. There was a similar fall in the activity of rabbit serum, after heating and zymosan absorption, against all antigens except starch and *Shigella flexneri*. The sera of some of the rabbits tested did however show up to a 4-fold fall in titre against these 2 antigens on zymosan absorption. In both guinea-pig and rabbit sera the fall in titre on zymosan absorption was very closely paralleled by the fall in titre on heating the sera at 55° for 20 min.

However, on absorbing human serum with 100 mg./ml. zymosan twice for 1 hr. at 4°, the immune-adherence titre fell only two-fold against all the antigens tested except *Ery. rhusiopathiae*, where the fall was four-fold ; absorption was not increased at 22°. There was no relation between fall in immune-adherence titre after zymosan absorption and after heating at 55° for 20 min. ; heating decreased the titre up to 16-fold, but zymosan absorption only 2-fold.

Fractionation of the human serum (Table II) into euglobulin and pseudo-globulin showed that the immune-adherence activity of the serum against all the antigens tested except starch was divided between the 2 fractions. Activity against starch was completely in the euglobulin. Fractionation of the same serum after absorption with zymosan showed a marked fall in the activity against all the antigens except starch in the euglobulin, but no fall in the activity in the pseudoglobulin. The activity against starch in the euglobulin showed a slight rise in titre after zymosan absorption. In other human sera there was a marked fall in the euglobulin titre against starch after absorption with zymosan and in one of them activity in the euglobulin against *Sh. flexneri* was not reduced on absorption of the serum with zymosan.

TABLE II.—*The Effect of Fractionation of Guinea-pig and Human Serum on the Titre* (1 :) *Before and After Absorption with Zymosan*

	Guinea-pig				Human			
	Unabsorbed		Zymosan absorbed		Unabsorbed		Zymosan absorbed	
	Euglobulin	Pseudoglobulin	Euglobulin	Pseudoglobulin	Euglobulin	Pseudoglobulin	Euglobulin	Pseudoglobulin
Salm. typhi	64	<8	8	<8	8	64	<4	64
Sh. flexneri	32	16	<8	16	32	16	8	16
Bact. coli	32	16	<8	16	128	32	4	32
V. el tor	8	<8	<8	<8	16	64	4	64
Staph. aureus	32	16	<8	16	16	16	<4	16
Ery. rhusiopathiae	16	32	<8	32	8	8	<4	8
Starch	32	<8	<8	<8	32	<8	256	<8

Similar fractionation of guinea-pig sera showed that the greater part of the activity against all the antigens except *Ery. rhusiopathiae* was in the euglobulin. Activity against *Ery. rhusiopathiae* was present in both fractions. Whereas the activity in the euglobulin against all the antigens tested was reduced by absorption with zymosan, that in the pseudoglobulin fraction was unaffected.

Effect of absorption of normal guinea-pig serum with bacteria and polysaccharides

Absorption with bacteria (Table III) had an effect similar to absorption with zymosan, producing a marked fall in titre against all antigens except *Ery. rhusiopathiae*. Activity against *Ery. rhusiopathiae* in guinea-pig sera was reduced in titre only by absorption with *Ery. rhusiopathiae* itself.

TABLE III.—*Titres* (1 :) *of Immune-adherence Activity of Normal Guinea-pig Serum After Absorption with Bacteria*

		Serum absorbed with					
	Serum‡ unabsorbed	*Salm. typhi*	*Bact. coli*	*Sh. flexneri*	*V. el tor*	*Staph. aureus*	*Ery. rhusiopathiae*
Starch	256	16	32	32	16	<8	<8
Salm. typhi	128	<8	<8	<8	<8	<8	<8
Bact. coli	256	32	<8	32	32	16	<8
Sh. flexneri	64	16	16	<8	16	<8	<8
V. el tor	32	<8	8	8	<8	<8	<8
Staph. aureus	512*	32	—	—	—	<8	<8
	256†	—	8	<8	16	—	—
Ery. rhusiopathiae	256	256	256	256	256	256	<8

* Pool IV.
† Pool V.
‡ Pool II unless otherwise indicated.

Absorption of normal guinea-pig serum with bacterial cell walls prepared by shaking a thick bacterial suspension with glass beads in a Mickle tissue disintegrator and then treating the residue with crystalline trypsin and pepsin (Cummins and Harris, 1956) produced falls in titre similar to that found after absorption with whole bacterial cells (Table IV).

TABLE IV.—*Fall in Titre* (1 :) *of Immune-adherence Activity of Normal Guinea-pig Serum following Absorption with Bacterial Cell Walls*

		Absorbed with cell walls of :		
	Unabsorbed	*Salm. typhi*	*Sh. flexneri*	*V. el tor*
Salm. typhi . .	128 .	<8	16	16
Sh. flexneri . .	128 .	16	8	16
V. el tor . .	128 .	<8	8	<8

The fall in immune-adherence titre of normal guinea-pig serum against *Salmonella typhi*, *Sh. flexneri* and *Vibrio. el tor*, when absorbed with whole bacterial cells or bacterial cell walls of *Salm. typhi*, *Sh. flexneri* and *V. el tor*, was found to be associated with a similar fall in the bactericidal titre of the serum against these 3 organisms. The bactericidal titrations were performed by the method of Nagington (1956) at the same time as the immune-adherence titrations.

Table V shows the effect on the immune-adherence activity of normal guinea-pig serum against the same range of antigens after treatment with various polysaccharides. Apart from yeast mannan all these polysaccharides, despite their very marked differences in chemical structure, produced a significant fall in titre of immune-adherence activity against all the antigens except *Ery. rhusiopathiae*. The 4-fold fall in titre against *Salm. typhi* and *Bacterium coli* and the 2-fold fall in titre against *Sh. flexneri*, *Staphylococcus aureus* and starch were consistent with 2 batches of mannan, the second being treated with α amylase to remove any possible trace of glycogen.

Similar treatment of normal guinea-pig serum with 200 mg./ml. of glucose and 200 mg./ml. of crystalline bovine plasma albumin for 2 hr. at 4° had no

TABLE V.—*Titres* (1 :) *of Immune-adherence Activity of Normal Guinea-pig Serum following Treatment with Polysaccharides*

		Serum treated with									
	Serum untreated	Starch	Amylose	Glycogen	Dextran	Laminarin	Levan	Inulin	Xylan	Agar agar	Yeast mannan
Starch . . .	256* .	16	<8	<8	—	—	—	—	—	—	—
	512 .	—	—	—	64	8	<8	32	64	32	256
Salm. typhi . .	512 .	16	8	<8	—	—	—	—	—	—	—
	512 .	—	—	—	64	8	<8	64	32	32	128
Sh. flexneri . .	128 .	64	<8	<8	—	—	—	—	—	—	—
	256 .	—	—	—	64	32	8	32	8	64	64
Bact. coli . .	512 .	32	8	16	—	—	—	—	—	—	—
	512 .	—	—	—	64	32	<8	128	16	32	128
V. el tor . . .	64 .	16	<8	<8	—	—	—	—	—	—	—
	64 .	—	—	—	16	32	<8	8	<8	32	64
Staph. aureus . .	1024 .	64	16	64	—	—	—	—	—	—	—
	512 .	—	—	—	64	64	64	128	128	64	256
Ery. rhusiopathiae .	512 .	512	256	512	—	—	—	—	—	—	—
	1024 .	—	—	—	1024	1024	1024	1024	1024	1024	1024

* The upper figure of each pair represents Pool III and the lower Pool IV.

effect on the immune-adherence activity of the serum against *Salm. typhi*, *Sh. flexneri*, *Bact. coli*, *V. el tor*, *Staph. aureus*, *Ery. rhusiopathiae* and starch.

Mackie and Finkelstein (1930) suggested that the absorption of non-specific complement-fixing factor of normal serum by bacteria was similar to its absorption onto charcoal. Charcoal, however, was found to reduce the specific immune-adherence activity of normal guinea-pig serum against *Ery. rhusiopathiae* to the same extent as it reduced the non-specific activity against other antigens.

Effect of temperature on the absorption of immune-adherence activity by zymosan

In previous experiments sera had been absorbed with 200 mg./ml. of zymosan at a temperature of 4°. This appeared to remove the major part of the non-specific immune-adherence activity of normal sera. Properdin, however, has been shown to be removed better at 10° than at 5° when absorbed with 4·0 mg./ml. of zymosan (Leon, 1957).

The absorption of the non-specific immune-adherence activity of normal guinea-pig serum, as shown against *Salm. typhi*, with 4·0 mg./ml. of zymosan was also more efficient at 22° than at 4°. At 4° only a two-fold fall in titre from 128 to 64 occurred, whereas at 22° it had dropped significantly to 8.

The serum of a normal guinea-pig contains a heat labile non-specific activity against zymosan to titre 32. After immunization with 60 mg. zymosan given intraperitoneally in divided doses over 2 weeks, the titre was found to have risen to 128, falling to 64 only on heating at 55° for 20 min. Absorption of this immune serum with 4·0 mg./ml. zymosan for 2 hr. at 22° reduced the titre to less than 8. Absorption at 4° reduced the titre only to 32. However, this activity was found to be completely heat labile. It therefore appears that the heat stable immune antibody is absorbed equally well at 22° and at 4°. After the heat stable immune antibody has been absorbed at 4°, the heat labile non-specific activity which is present in the normal serum can be shown to be still present as it is not so efficiently absorbed at 4° as at 22°.

Immune-adherence activity of the normal serum of young animals

Immune-adherence activity was found in the serum of newborn calves, bled before they had been allowed to suckle and receive maternal antibody. Table VI

TABLE VI.—*Titres of Immune-adherence Activity of Serum of Young Animals*

(a) *Calf*—serum taken before suckling

Antigen	Fresh	Heated 55° for 20 min.	Absorbed with 0·1 g./ml. zymosan twice at 4°
Salm. typhi	32	<8	<8
Staph. aureus	64	<8	<8
Starch	128	<8	<8

(b) Rabbit—titres of activity against starch

	Newborn before suckling	One month old	Two months old
(i) Fresh	1024	128	512
(ii) Heated 55° for 20 min.	1024	<8	512
(iii) Absorbed with 0·1 g./ml. zymosan twice at 4°	1024	<8	512

shows the activity against *Salm. typhi*, *Staph. aureus* and starch. Activity to titre 32 was found against *Salm. typhi*, 64 against *Staph. aureus* and 128 against starch. The activity against these 3 antigens was destroyed by heating at 55° for 20 min. and completely removed by absorption with zymosan.

Serum from newborn rabbits bled before suckling, however, had a titre of 1024 against starch which did not fall on absorption with zymosan or on heating at 55° for 20 min. This activity is the same as that of adult serum and is in keeping with the observations of Brambell *et al.* (1948) that antibody passes from the rabbit to its foetus by the maternal circulation in utero, whereas antibody is transmitted by the cow to its calf by the colostrum. In one-month-old rabbits the immune-adherence titre against starch had dropped to 128 ; it was completely removed by absorption with 0·1 mg./ml. zymosan or inulin twice at 4° ; or by heating at 55° for 20 min. By 2 months the rabbits had developed the heat stable specific antibody against starch which was unaffected by zymosan absorption.

Effect of immunization on the non-specific immune-adherence activity of normal serum

The effect of an injection of zymosan, levan or bacterial polysaccharide in reducing natural immunity has been shown by Pillemer *et al.* (1955) to be associated with a reduction in the level of properdin. The titre later rose to a maximum in 5–6 days and then fell to pre-inoculation level within 14 days.

The Figure shows the change in immune-adherence titre of a normal adult rabbit following a single injection of 2×10^9 *Salm. typhi* 0901 intravenously. Sera were taken hourly for the first 8 hr. and then after 24 hr. Following this, sera were taken every 2 days for 2 weeks and then weekly. The sera were tested for immune-adherence activity against *Salm. typhi* 0901, *Salm. typhi* 540 (rough), *Sh. flexneri* and *V. el tor*. The response was of 2 types, a specific rise in titre to *Salm. typhi* 0901 and a non-specific rise in titre to the rough strain of *Salm. typhi*, *Sh. flexneri* and *V. el tor*. It was preceded by a fall in titre against all 4 organisms, lowest 7 hr. after injection. The specific response reached its peak about 7 days after injection and took more than 3 months to fall back to the pre-inoculation level. The non-specific response was much less ; it also reached a peak 7 days after injection, but fell to the pre-inoculation level after about 14 days. Essentially the same kinds of specific and non-specific response were produced by the intravenous inoculation of 2×10^9 *Sh. flexneri*, a specific type of response was demonstrated against *Sh. flexneri*, and a non-specific response was also found against *Salm. typhi* 0901 and *V. el tor*. A non-specific response curve against *Salm. typhi*, *Sh. flexneri* and *V. el tor* was also produced by the intravenous injection of 60 mg. of zymosan into a rabbit.

DISCUSSION

The immune-adherence phenomenon provides a very sensitive technique for the investigation of antibody and antibody-like activities of normal sera upon bacteria and particulate polysaccharide antigens. It has the advantage over bactericidal titrations in that it does not appear to depend on the sensitivity of the bacteria used. For example, in immune-adherence titrations normal sera may give similar titres with both smooth and rough variants of the same organism,

whereas the rough variant may be very much the more sensitive to the bactericidal action of the same serum. Bactericidal titrations are also limited to Gram negative organisms and the *Bacilli* ; immune-adherence on the other hand takes place also with Gram positive organisms insensitive to the bactericidal activity of serum and with particulate polysaccharide antigens.

Two types of activity can be distinguished in normal sera by the immune-adherence of bacteria and polysaccharide antigens. There appears to be a basic non-specific activity against all bacteria and polysaccharides so far tested, and a

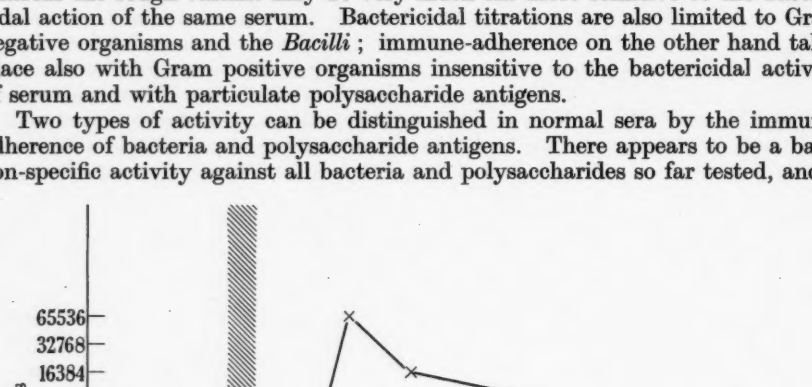

FIGURE.—Immune-adherence titres in rabbit serum following the intravenous injection of 2×10^9 *Salmonella typhi* 'O' 901.

× —— × *Salm. typhi* 'O' 901.
○ - - - ○ *Th Flexneri* 4.
● —— ● *V. el tor.*
▲ *Salm typhi* (rough).

secondary specific activity against individual antigens which may be superimposed on the first to a varying degree.

The non-specific activity has many properties in common with that of properdin (Pillemer *et al.*, 1955). It was found to be absorbed by a large range of bacteria and by a number of polysaccharides of widely different stereochemical structure. These may contain α or β linkages, furanose or pyranose rings with 1 : 4, 1 : 6, 1 : 3, 2 : 1, or 2 : 6 linkages. It is also characteristic of properdin, as of the heat labile non-specific immune-adherence factor, that its absorption is more efficient at 22° than at lower temperatures (Leon, 1957), whereas classical antibody is absorbed equally well at 4° and 22°. The non-specific activity of normal serum as demonstrated by immune-adherence appears to be in the euglo-

bulin as does properdin (Pillemer *et al.*, 1954). The parenteral injection of bacteria or zymosan produces a fall in the non-specific immune-adherence activity of serum within the first few hours after injection, followed later by a rise in activity reaching a maximum 7 days after injection and falling to the pre-inoculation level within 14 days. This resembles the initial fall and subsequent rise in the level of properdin described by Pillemer *et al.* (1955) as occurring after the injection of zymosan or bacterial polysaccharides.

Whereas the non-specific activity of normal serum is heat labile when the serum is heated at 55° for 20 min., the specific antibody-like activities of guinea-pig and rabbit sera are heat stable under similar conditions. In human sera, however, the titre is only partially reduced by similar treatment. The degree of specificity is illustrated by the fact that the level of antibody against starch in normal adult rabbit serum is not reduced by absorption with zymosan, whose major component is a glucan differing from starch only in that its glucose molecules are joined by β 1 : 3 and β 1 : 6 instead of α 1 : 4 and α 1 : 6 linkages as they are in starch. This degree of specificity is similar to that found with enzymes acting on these compounds.

In most cases this specific activity is lower in titre than the non-specific activity, though in some cases it is far higher. This is particularly striking with the activity of adult rabbit sera against starch and with the activity of guinea-pig sera against *Ery. rhusiopathiae*. The specific activity is present mainly in the pseudoglobulin fraction, whereas the non-specific activity is in the euglobulin.

The specific immune-adherence activity of normal serum appears to develop with age. It is absent in the newborn calf before suckling and in the one-month-old rabbit. Specific activity against starch develops by the age of 2 months in the rabbit. This is similar to the development of antibody against *Trichomonas* in the calf (Pierce, 1955). Non-specific properdin-like immune-adherence activity is present in the newborn calf and in the one-month-old rabbit.

The simplest explanation of these observations is that Mackie and Finkelstein's (1930) non-specific complement-fixing activity, Pillemer's properdin (1954), and the heat labile non-specific immune-adherence factor in normal sera are manifestations of the same mechanism, and that the specific activities are true antibodies also present in normal sera.

SUMMARY

Immune-adherence has been shown to occur with normal guinea-pig, rabbit and human sera against *Salm. typhi*, *Sh. flexneri*, *Bact. coli*, *V. el tor*, *Staph. aureus*, *Ery. rhusiopathiae* and starch. The total activity appears to be the resultant of a non-specific activity and a specific activity against the particular antigen. The non-specific activity is in the euglobulin whereas much of the specific activity is in the pseudoglobulin. The non-specific activity is absorbed by a wide range of polysaccharides and has many properties in common with properdin, and seems closely related to the complement-fixing activity in normal serum described by Mackie and Finkelstein (1930). It is present in the serum of the newborn calf before suckling and in the suckling rabbit. The specific activity appears to be antibody and though absent in the one-month-old rabbit has developed by the second month.

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THE INFLUENCE OF SODIUM FLUOROACETATE ON REGENERATION OF THE RAT'S LIVER

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SINCE the recognition of the interference in citrate metabolism produced by fluoroacetate (Liebecq and Peters, 1949; Martius, 1949), several reports have appeared describing the use of this compound as an indicator of the tricarboxylic acid cycle (Buffa and Peters, 1949; Potter, Busch and Rothwell, 1951; Peters, Wakelin, Buffa and Thomas, 1953). The most widely held view relating to the mode of action of fluoroacetate, attributes the toxic effects to an inhibition of aconitase by fluorocitrate, synthesised *in vivo* from fluoroacetate (Peters, 1957), and the effect of aconitase blocking would theoretically inhibit the energy-yielding reactions associated with the Krebs cycle after citrate→isocitrate conversion. Biological effects of fluoroacetate administration have been described in pigeons (Peters, 1952) its activity as a mitotic inhibitor has been tested *in vitro* (Hughes, 1950) and an evaluation of fluoroacetate as a carcinostatic agent has been attempted (Dietrich and Shapiro, 1956).

Bullough and Johnson (1951) postulated that mitotic activity in the mouse epidermis related to tricarboxylic acid energy production and Bullough (1952) considered it possible that other mammalian tissues which develop a high mitotic rate followed a similar physiological plan. One such tissue is the liver which has a remarkable ability to replace lost tissue by cellular hypertrophy and hyperplasia. Following partial resection the liver remnant shows a roughly exponential growth rate for the first 3 days (Doniach and Weinbren, 1952) and a high rate of mitoses beginning in the first 24–30 hr. (Harkness, 1957). The energy relationships and chemical reactions involved in this process are not known but it is likely that both the phases of growth and division are greatly dependent on the various energy-yielding reactions taking place in the cells (Swann, 1957).

It seemed reasonable to attempt to assess whether liver regeneration was influenced by interference with the tricarboxylic acid cycle and the experiments to be described were designed to measure the effect on this process by fluoroacetate blocking.

MATERIAL AND METHODS

Rats.—Male and female albinos, bred in our laboratories and weighing 100–250 g. were fed on " Research " rat cubes. Food, but not water, was withheld from control and experimental animals after the injection of fluoroacetate in short-term (28 hr.) experiments. This was done because the animals invariably ate little or nothing after fluoroacetate injection and it seemed advisable to restrict the food intake of controls at the same time.

Sodium fluoroacetate.—This was kindly supplied by Associated Fumigators Ltd. (99 per cent pure), was made up 1 mg./ml. distilled water and the dose of 0·1–0·3 mg./100 g. body wt. was given by intraperitoneal injection.

Citrate levels in tissues.—This was estimated by the pentabromoacetone method described by Taylor (1953).

Incidence of mitoses was expressed per 1000 cells after 100–200 alternate high power fields (objective ×40, ocular ×6), using a square ocular limiting a field 0·16 mm. edge. The total number of cells were counted in every 10th field. In this way, a total of 5000–8000 cells were examined in each section.

The following experiments were designed to test the effect of fluoroacetate on the incidence of mitoses after partial hepatectomy.

Group I. Survival after intraperitoneal fluoroacetate

Removal of the anterior 2 lobes of the liver (approximately 2/3 partial hepatectomy) was carried out by the method of Higgins and Anderson (1931) in 48 rats. Fluoroacetate was given in doses of 0·3 mg./100 g. body wt. within 2 hr. of operation to 15 rats and to 12 unoperated controls, 0·2 mg./100 g. to 23 and 10 controls, and 0·15 mg./100 g. to 10 and 10 controls. The period of survival was taken as 30 hr.

Group II. Duration of activity of fluoroacetate after intraperitoneal injection

Partial hepatectomy was carried out in 30 males which were given 0·1 mg. fluoroacetate at operation and which were killed in groups of 7 or more after 4, 8, 16 and 24 hr. The renal citrate concentration has been shown to vary directly with the injected dose and that this increased level is constantly found after fluoroacetate. Changes in the hepatic citrate concentration on the other hand are equivocal, some authors reporting high levels (Ord and Stocken, 1953) which are not confirmed by others (Potter and Busch, 1950).

Group III. Determination of time of first wave of mitoses after partial hepatectomy

Livers were examined for mitotic figures from 9 animals killed in groups of 3 each, 22, 24 and 28 hr. after partial hepatectomy respectively.

Group IV. Effect of a single injection of fluoroacetate 8 hr. after partial hepatectomy on first wave of mitoses

Partial hepatectomy was performed in 22 males, fluoroacetate was injected into alternate animals 8 hr. later, after which food was withheld from both experimental and control animals and all were killed after 28 hr. Citrate levels were estimated in the kidneys and the livers were weighed and sections taken for microscopy.

Group V. Effect of a single late injection of fluoroacetate

A group of 12 rats were partially hepatectomized and after 24 hr. fluoroacetate was given to 6. All were killed after 28 hr.

Group VI. Effect of repeated injections of fluoroacetate

In 5 rats injections of the compound were given in doses of 0·1 mg./100 g. 8, 16, 24, 36, 48 and 72 hr. after partial hepatectomy and the rats were killed together with operated uninjected controls at 96 hr. A further 9 rats were given 0·15 mg. after 12, 24, 36 and 48 hr. and the survivors were killed after 3 days.

RESULTS

Survival after intraperitoneal fluoroacetate

The number of rats surviving injections of the higher doses of fluoroacetate was considerably reduced by partial hepatectomy (Table I). Doses of 0·3 mg./100 g. were lethal in 13 out of 15 partially hepatectomized rats and only in 2 out of 12 sham operated controls, and doses of 0·2 mg./100 g. were lethal in 8 out of 23 operated animals and only in 1 out of 10 controls. All the test animals survived injections of 0·15 mg./100 g. and the remaining experiments were carried out using this dosage or 0·1 mg./100 g. body wt.

TABLE I.—*Survival of Unoperated and Partially Hepatectomized Rats 24 hr. after Intraperitoneal Fluoroacetate*

Dose mg./100 g. body wt.	Unoperated		Partially hepatectomized	
	Number injected	Survivors	Number injected	Survivors
0·15	10	10	10	10
0·2	10	9	23	15
0·3	12	10	15	2

Duration of activity of fluoroacetate

The citrate concentration in the kidney is greatest during the first 4–8 hr. after a single injection. As Peters (1957) found, the level then falls, and after 24 hr. the citrate level is almost normal (Table II).

TABLE II.—*Renal Citrate Levels after 0·1 mg./100 g. Intraperitoneal Fluoroacetate*

Number of rats	Time after fluoroacetate (hr.)	Citrate level	
		μg./g. wet tissue	Range
6	Control	37	13–80
7	4	251	139–361
7	8	174	100–304
8	16	64	36–97
7	24	45	31–62

First mitoses after partial hepatectomy

Confirming previous data on rats of the same strain, only occasional mitoses were found 22 and 24 hr. after partial hepatectomy, although abundant divisions were present at 28 hr. In the fasted controls the incidence of mitoses was 18·3/1000 cells ±S.D.6·2.

Effect of intraperitoneal fluoroacetate

The incidence of mitoses was much reduced when the fluoroacetate was given 8 hr. post-operatively, but not if the injections were made 24 hr. after partial hepatectomy (Table III). The mean level of mitoses in the controls at

TABLE III.—*Incidence of Mitoses in Regenerating Liver after Intraperitoneal Fluoroacetate*

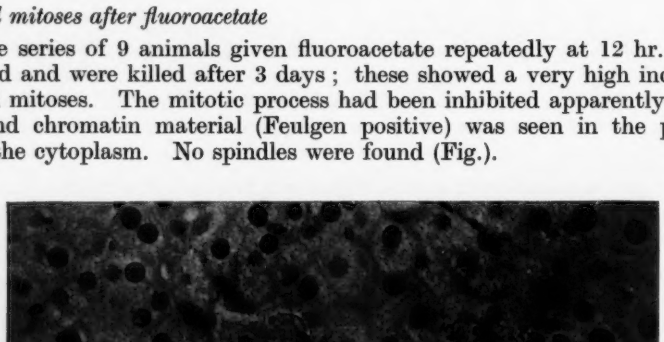
Number of rats	Time of injection (post-operative)	Time of death (hr.)	Mitoses/1000 cells	S.D.
11	Control (fasted)	28	18·3	±6·2
11	8 hr.	28	3·5	±2·6
6	24 hr.	28	25·4	±8·3
5	Repeated	96	19·9 (control 5·8)	±8·2

28 hr. is 18·3 ± S.D. 6·2 and that in the animals treated at 8 hr. is 3·5 ± S.D. 2·6. In one series, fluoroacetate was administered repeatedly for 3 days and the

incidence of mitoses was greater (19·9 ± S.D. 8·2) than that in controls (5·8 ± S.D. 2·1). At the same time it was found that fluoroacetate did not induce mitoses in unoperated controls.

Abnormal mitoses after fluoroacetate

In one series of 9 animals given fluoroacetate repeatedly at 12 hr. intervals 6 survived and were killed after 3 days; these showed a very high incidence of abnormal mitoses. The mitotic process had been inhibited apparently at metaphase, and chromatin material (Feulgen positive) was seen in the peripheral parts of the cytoplasm. No spindles were found (Fig.).



Section from rat liver, after repeated intraperitoneal injections of sodium fluoroacetate for 3 days, shows many disrupted mitotic figures; Feulgen stain. × 350.

Microscopical changes in the livers after fluoroacetate

Distinct morphological alterations were found in the liver cells; the cytoplasm showed a diffuse finely vacuolated appearance and there was considerable fat accumulation. This change, to a lesser degree, was found in control partially hepatectomised animals fasted before death. In addition there was considerable phagocytosis of haematoxyphil material in the Kupffer cells after administration of the agent and these alterations were not found in the starved untreated animals. In a few cases centrilobular necrosis was found after repeated administration of the compound to sham-operated animals. We did not find centrilobular necrosis in partially hepatectomized rats.

DISCUSSION

The results in this series of experiments showed that the incidence of mitoses was greatly reduced in regenerating liver tissue by fluoroacetate. This inhibitory

action was not effective if fluoroacetate was injected 24 hr. after operation and some 4 hr. before the first wave of mitoses, although at this time there was definite biochemical evidence of the blocking action on the Krebs cycle and the animals were clinically ill.

There are certain difficulties involved in interpreting the findings. It seems reasonable to accept that the action of the derived fluorocitrate is inhibitory to aconitase and that the increased tissue concentration of citrate may be taken as an indicator of the effectiveness of the administered compound (Peters, 1957). In the present experiments, the greatest effect of the blocking action, judging from the citrate levels, is during the 4–8 hr. following the injection. It is likely that citrate → isocitrate conversion is depressed then and the subsequent reactions in the tricarboxylic acid cycle affected. If this is a justifiable assumption and previously published data (Buffa and Peters, 1949; Potter and Busch, 1950; Peters, 1957) support this view, then we may infer that energy yielding reactions have been inhibited during this time. Thus it might be possible to suggest that mitoses have been inhibited as a result and that therefore the mitotic process in regenerating liver tissue may be dependent to a large extent on the reactions of the tricarboxylic acid cycle. If this is a valid interpretation, then it is of great interest that depression of the energy-yielding reactions immediately before and during the mitotic process was not as effective as depression beginning some 12–15 hr. previously. It is known that metabolic changes can be detected by alterations in RNA and DNA activity about 12 hr. after partial hepatectomy and therefore 12–15 hr. before the first wave of mitoses (Cater, Holmes and Mee, 1956) and that similar pre-mitotic activity is found in many other living tissues. Swann (1957) has referred to this phenomenon as a building up of a so-called energy reservoir so that the metabolic inhibition initiated immediately preceding and during the mitotic wave might be expected not to depress the incidence of mitosis. This concept of the energy reservoir related to the development of mitosis would seem to accord with the results in mammalian liver tissue as well as in unicellular organisms.

There were significant alterations mainly in the cytoplasm which were visible by light microscopy at the dose levels employed, but no evidence of necrosis at sublethal dosages, and no wave of mitotic figures after administration of the compound in unoperated animals. In the long-term series, therefore, when the treated partially hepatectomised animals showed a higher incidence of dividing cells than the controls, the most reasonable interpretation is that the effect of fluoroacetate is competitive in this context and that the reactions may be restored after some time. In this way the appearance of mitosis may be delayed because of the temporary inhibition.

Whether the action of the compound is directly on the liver tissue or by some mechanism secondary to disordered function elsewhere is difficult to resolve. Whilst it is known that fluoroacetate exerts a definite action on heart muscle, among other organs, it might be concluded that the effect on liver mitoses may have been secondary to reduced blood-flow. Stoner (1956) measured the liver blood flow using the thermocouple method described by Grayson (1952), and concluded that even when hepatic necrosis was induced by fluoroacetate there appeared to be no reduction in hepatic blood flow. The evidence suggests the possibility of a direct action on liver cells and that as a result of this mechanism, mitoses in regenerating liver tissue are inhibited.

SUMMARY

Sodium fluoroacetate in sublethal doses, was injected into white rats at various times after partial hepatectomy. The incidence of hepatic mitoses 28 hr. after resection was decreased when the fluoroacetate was given 8 hr. after operation but not at 24 hr. Repeated small doses of fluoroacetate resulted in an increase of mitoses after 4 days. In one series of rats, receiving repeated higher doses, abnormal mitoses developed.

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A COMPARATIVE SURVEY OF THE PHAGOCYTOSIS OF DIFFERENT SPECIES OF BACTERIA BY KUPFFER CELLS.

Perfusion Studies with the Isolated Rat Liver

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A technique has been described recently for measuring the phagocytosis of viable bacteria by Kupffer cells during perfusion of the isolated rat liver (Howard and Wardlaw, 1958). An opsonic effect of normal serum in this system was demonstrated by the increased rate of phagocytosis of *Bacterium coli* when suspended in serum as compared with Ringer-Locke, both suspensions being passed successively through the same liver. The serum factors which promoted phagocytosis appeared to be specific antibody, complement and another heat-labile factor which was absorbed by zymosan. The identity of the latter opsonin with properdin was suggested but not established satisfactorily, primarily because of the superimposed effect of antibody in most of the sera tested. We have accordingly examined a range of bacterial species in an attempt to find a more suitable organism for which the opsonic activity of serum would be entirely heat-labile. This investigation has produced some unexpected findings, for not only were certain bacteria phagocytosed extremely well when suspended in Ringer-Locke, but serum in fact reduced the percentage uptake of such strains. The results of these experiments will be described here.

MATERIALS AND METHODS

Bacterial strains

The following were used: *Bacterium friedländeri* (strain 2F), *Salmonella gallinarum* (strain 9604), *Bacterium alkalescens* (strain "Inbry"), *Bacterium coli* (strain 145), *Pseudomonas pyocyanea* (strain 210), *Proteus mirabilis* (strain "Graves"), *Staphylococcus aureus* (strain "Oxford"), *Corynebacterium murium* (N.C.T.C. No. 1386), *Bacillus cereus* (strain CN 2504), *Streptococcus pyogenes* Lancefield group C (strain CN771), *Clostridium welchii* type A (W.F.I. strain), *Clostridium oedematiens* (W.F.I. strain) and *Streptococcus pneumoniae* type 1 (N.C.T.C. No. 7465). These strains were selected as representative of a wide variety of morphological types. They all satisfied the requirements of being stable in Ringer-Locke suspension at room temperature and 37° for the periods required and of showing no reduction in viable count on exposure to human serum under the conditions used.

Human Serum

One batch of pooled serum was used throughout. It was obtained from 8 Group O donors and was stored at −20°.

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Rats

Albino animals of either sex and weighing 200–300 g. were used. In any single experiment rats of the same sex were used, with weights varying by not more than 20 g.

Preparation of bacterial suspensions

The suspensions were prepared from overnight 37° nutrient broth cultures. *Staph. aureus*, *B. cereus* and *Str. pyogenes* were grown in a mechanical shaker to obtain a dispersed growth, whilst *Cl. welchii* and *Cl. oedematiens* were grown in a McIntosh and Fildes jar. All cultures were centrifuged before use and the deposited bacteria resuspended in Ringer-Locke to a density equivalent to approximately 5×10^8 viable units/ml. These suspensions were kept at room temperature and used within 1½ hr. Perfusion was done with fluids containing approximately 10^5 viable units/ml., the bacteria being added from the 5×10^8 suspension to the warmed (39°) fluids as required.

Perfusion Technique

The procedure previously described (Howard and Wardlaw, 1958) was used. It involved cannulating the portal and hepatic veins, washing the liver free of blood and perfusing it successively with bacterial suspensions in Ringer-Locke, heated serum and unheated serum. The sera were used at 25 per cent concentration in Ringer-Locke. The apparatus was such that flow through the organ was not interrupted when changing from one suspension to the next. Experiments were completed within 10–12 min. of the death of the animal.

Estimation of percentage phagocytosis

The extent of phagocytosis was estimated as previously from the difference in numbers of viable bacteria per ml. in fluids before and after passage through the liver. Viable counts were made using nutrient agar as the plating medium except with *C. murium*, *Str. pyogenes*, *Str. pneumoniae*, *Cl. welchii* and *Cl. oedematiens* when blood agar was used. Plates with clostridia were incubated anaerobically. In each liver the rate of uptake of bacteria from Ringer-Locke was compared with that from heated and unheated serum. At least 3 animals were used with each strain and mean uptake percentages in Ringer-Locke and heated and unheated serum obtained.

RESULTS

The susceptibilities of 6 Gram-negative and 7 Gram-positive strains to phagocytosis by Kupffer cells, and the influence of heated and unheated serum on this process are shown in the Table. Several distinct patterns of behaviour are apparent in these results.

With 3 of the Gram-negative strains (*Bact. friedländeri*, *Salm. gallinarum* and *Bact. alkalescens*) no significant phagocytosis was detected using suspensions in Ringer-Locke. On the other hand, *Bact. coli*, *Ps. pyocyanea* and *Pr. mirabilis* were clearly taken up, although to different extents, from this medium. The suspensions of all 6 Gram-negative strains in unheated serum demonstrated a clear opsonic effect, whilst the 2 patterns of behaviour observed in Ringer-Locke were no longer obtained. Previous heating of the serum at 56° for 30 min. lowered its opsonic activity for all strains. In the case of *Pr. mirabilis* the reduction was slight, whilst with *Salm. gallinarum*, the opsonin appeared to be entirely thermolabile.

The 7 Gram-positive strains did not behave in such a uniform manner. Especially striking was the very high uptake (near 85 per cent) of *Staph. aureus*, *C. murium*, *B. cereus* and *Str. pyogenes* from suspensions in Ringer-Locke. When these 4 strains were suspended in either heated or unheated serum, the percentage uptakes were reduced (means of 61 and 64 respectively). A different result was obtained with the 2 clostridia, which resembled some of the Gram-negatives in

TABLE.—*The Susceptibility of Various Bacteria to Phagocytosis by Kupffer Cells and the Influence of Heated and Unheated Normal Human Serum on this Process*

	Mean percentage removal of bacteria from suspension in		
	Ringer-Locke	Heated serum†	Unheated serum
Gram-negative bacteria—			
Bact. friedländeri	3	32	53
Salm. gallinarum	0	0	53
Bact. alkalescens	0	45	62
Bact. coli	11*	27*	41*
Ps. pyocyanea	35	47	74
Pr. mirabilis	48	62	69
Gram-positive bacteria—			
Staph. aureus	85	54	67
C. murium	79	54	58
B. cereus	88	68	75
Str. pyogenes	86	66	54
Cl. welchii	0	12	36
Cl. oedematiens	4	70	87
Str. pneumoniae	0	0	0

* Mean of 9 animals.
† 56° for 30 min.
The uptake figures for each strain are the means from three rat's livers each of which was perfused successively with organisms suspended in Ringer-Locke, 25 per cent heated serum and 25 per cent unheated serum. A difference of 10 in mean percentage uptake is significant ($P = 0{\cdot}05$) and a difference of 13, highly significant ($P = 0{\cdot}01$).

having a negligible uptake from Ringer-Locke that was greatly enhanced by both thermostable and thermolabile opsonins in serum. *Str. pneumoniae* differed yet again in being phagocytosed from neither Ringer-Locke nor serum.

DISCUSSION

Some of these observations are in accord with and extend the comparatively early work of Manwaring and his colleagues (Manwaring and Coe, 1916; Manwaring and Fritschen, 1923). Using a liver perfusion technique in rabbits and dogs, these authors found a high (80 per cent) uptake of *Staph. aureus* by Kupffer cells from Ringer-Locke suspensions. *Str. pneumoniae* was not phagocytosed when suspended in either Ringer-Locke or normal serum, but dilute immune serum was highly opsonic. The very efficient clearance of 4 Gram-positive strains when perfused without serum, which we have observed, is clearly at variance with most previous experiments using free phagocytes. For example, neither *Str. pyogenes* nor *Staph. aureus* were phagocytosed appreciably by polymorphs *in vitro* when suspended in saline (Hektoen and Ruediger, 1905; Nanni, 1958). Lucké, Strumia, Mudd, McCutcheon and Mudd (1933) found that in the absence of serum, exudate macrophages would take up *Myco. tuberculosis* var. hominis, but not *B. subtilis* or *Bact. coli*.

An attempt may be made to correlate our findings using Ringer-Locke suspension with the morphology of the respective bacteria. Thus the 3 flagellated

Gram-negative strains (*Bact. coli*, *Ps. pyocyanea* and *Pr. mirabilis*) were phagocytosed, whilst the three non-flagellated strains (*Bact. friedländeri*, *Bact. alkalescens* and *Salm. gallinarum*) were not. The resistance of *Str. pneumoniae* and *Cl. welchii* might be associated with the possession of capsules, although *Cl. oedematiens* is both non-encapsulated and flagellated. It is possible that the very high uptake of the remaining 4 Gram-positive strains may be determined by some feature of their surface such as a high negative charge. Gram-positive strains in general are known to have a lower iso-electric point than Gram-negative bacteria when suspended in saline (Mudd, McCutcheon and Lucké, 1934).

Much smaller inter-strain differences in susceptibility to phagocytosis were observed when serum was present in the suspending medium. Excluding *Str. pneumoniae* which appears to require immune serum for opsonization, the mean uptake of 6 Gram-negatives was 59 per cent and the mean of 6 Gram-positives was 63 per cent. When variations in the opsonin content for different strains in one batch of serum are considered, the degrees of phagocytosis of these strains are not dissimilar. These findings might be explained by the effect of opsonin on bacterial surface charge. Mudd *et al.* (1934) found that the iso-electric points of 6 different organisms in saline varied over the range pH 2·6–3·8. In the presence of increasing amounts of immune serum, the iso-electric points all converged to pH 5·0. With normal serum, the convergence point was 4·6. It seems likely that if all bacteria are equally effectively coated with opsonin, they would be treated in a similar manner by the R.E.S. irrespective of basic differences in the organisms themselves. Clearly, the usual result of serum treatment is promotion of phagocytosis, although with certain strains which are very effectively taken up in Ringer-Locke, the reverse effect may be obtained.

SUMMARY

The susceptibilities of 6 Gram-negative and 7 Gram-positive bacterial species to phagocytosis by Kupffer cells and the influence of heated and unheated normal human serum have been studied by a rat liver perfusion technique.

The 3 flagellated Gram-negative strains (*Bact. coli*, *Ps. pyocyanea* and *Pr. mirabilis*) were phagocytosed in Ringer-Locke solution whilst the 3 non-flagellated Gram-negative strains (*Bact. friedländeri*, *Salm. gallinarum* and *Bact. alkalescens*) were not. All 6 were taken up to approximately the same extent in normal serum, which exerted an opsonic effect in each instance. Heating the serum at 56° reduced its opsonic activity towards all 6 strains, and in the case of *Salm. gallinarum* abolished it completely.

Four of the Gram-positive strains (*Staph. aureus*, *B. cereus*, *C. murium* and *Str. pyogenes*) were very readily phagocytosed from Ringer-Locke and the effect of heated and unheated serum was to reduce the uptake. Two clostridia behaved similarly to the Gram-negative group, while a capsulated pneumococcus was not phagocytosed from either Ringer-Locke or serum suspensions.

We wish to thank Dr. D. Rowley, in whose department this work was done, for his valuable help and advice. We are indebted to the members of the general public who gave blood and Dr. J. D. James of the North London Blood Transfusion Centre, who made the necessary arrangements. The competent technical assistance given by Miss M. Ham is gratefully acknowledged.

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ANTIBODY PRODUCTION BY SINGLE CELLS: II. THE DIFFERENCE BETWEEN PRIMARY AND SECONDARY RESPONSE

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ONE of the unsolved problems in immunology is the nature of the difference between the primary and the secondary response. In a secondary response, far more antibody is formed; this could be due to more cells producing antibody, to individual cells producing more antibody, or to a combination of these 2 factors. Burnet's (1957) clonal selection hypothesis of antibody formation envisages selective multiplication of clones of antibody-producing cells following antigenic stimulation. The secondary response could then be explained by the presence of a larger number of cells from the antibody-producing clone, resulting in a larger total output of antibody following further antigenic stimulation.

In previous communications (Nossal and Lederberg, 1958; Nossal, 1958) a technique was described for the detection of antibody production by single cells from hyperimmunized animals. This technique depended on the specific immobilization of *Salmonella* organisms by antiflagellar antibody. In the present experiments, the antibody output of isolated cells removed at the peak of either the primary or the secondary response was studied. It was shown that the chief difference between the primary and the secondary response was in the proportion of cells actively forming antibody.

MATERIALS AND METHODS

Animals.—Adult male Wistar albino rats were used. They were fed on a diet of cubes and tap water *ad libitum*.

Bacteria.—The monophasic organism *Salmonella adelaide*, flagellar antigen H_1 ^{fg}, was used. Bacteria were maintained at maximal motility by frequent passages through a semi-solid nutrient gelatin agar medium (Lederberg, 1956). Fresh 4 hr. broth cultures were used for motility-inhibition tests.

Antigen.—The antigen used was a preparation of isolated flagella from *Salm. adelaide*. The preparation of this antigen will be described in detail elsewhere. In summary, bacteria grown for 40 hr. on nutrient agar were suspended in saline, homogenized in a Waring blender for 3 min., and removed by centrifugation. The flagella present in the supernatant were deposited by high speed centrifugation in a Spinco ultracentrifuge. About 0·5 ml. of flagellar material was dissolved in 10 ml. of sterile physiological saline, and this solution was termed flagellar antigen.

Immunization of animals.—Rats were immunized by injecting 0·25 ml. of a 1 : 100 dilution of flagellar antigen in physiological saline into each hind foot-pad. Animals used for study of the primary response were killed 7 days after such injections. For the study of the secondary response, animals were similarly inoculated 4–6 weeks after the primary injections, and killed 6 days later.

Preparation of cell suspensions and micromanipulation.—The techniques used for the preparation of single cell suspensions from the popliteal lymph nodes draining the injection site

and for the isolation of these cells in microdroplets have been described in detail (Nossal, 1958). Several inprovements in technique have since been made, which are included in the following abbreviated description.

Both popliteal nodes from one animal were pooled, teased with needles in a Petri dish containing Earle's saline buffered to pH 7·0 with tris (hydroxy-methyl) amino methane (TRIS, Sigma & Co.), forced through a coarse sieve, and gently washed 3 or 4 times in Earle's saline by centrifugation. The final deposit of cells was suspended in sufficient Earle's saline supplemented with 30 per cent normal rat serum to make the concentration of cells approximately 10^6 per ml. In the studies of the primary response, this cell suspension was used for preparing microdroplets by free-hand manipulation. A coverslip was covered with medicinal paraffin oil, B.P. (H.C. Sleigh & Co.) and by the use of a fine Pasteur pipette, droplets containing 0 to 10 cells were deposited on the surface of the coverslip. The paraffin oil minimized evaporation. Such droplets contained about 10^{-5} to 10^{-6} ml. of fluid. The coverslip was then inverted over a de Fonbrune (1949) oil-chamber and incubated at 37° for 4 hr. In the secondary response studies, the final cell suspension was diluted to a concentration of approximately 2×10^4 cells per ml. with suspending medium and a large depot droplet, containing several hundred cells, was deposited on the coverslip. Numerous large droplets containing suspending medium only were also deposited. The coverslip was then inverted over the oil-chamber, and by micromanipulation under 100-fold magnification (dark-ground), single cells were drawn into the micropipette from the depot drop, washed in a clean droplet of suspending medium, and deposited on a known spot on the coverslip. Beside each droplet containing a single cell, a droplet of the cell's final washing fluid was placed for control purposes. About 40 such pairs of droplets could be prepared in about 1½ hr. The oil-chamber was then incubated at 37° for 4 hr.

The oil chamber was then placed on the microscope-stage, and a " cloud " of bacteria was inoculated into each droplet by micromanipulation. Owing to the large number of droplets present in each experiment, the exact number of bacteria in this cloud was not counted. but with increasing skill of the operator, rarely contained less than 7 or more than 10 bacteria. After 30 min. at room temperature, the droplets were surveyed for motility of the bacteria. Total loss of motility of all the bacteria was recorded as " inhibition ".

Titration of antibody content of microdroplets.—Droplets containing single cells showing inhibition were inoculated with a further cloud of bacteria, and observed at 5–10 min. intervals. As the number of active single cells in any experiment was small, greater accuracy was achieved here, and the number of bacteria introduced was actually 7–10. If at any time in the next 30 min. total immobilization of the bacteria had occurred, a third lot of 7–10 bacteria was introduced, and so on until the antibody content of the droplet had been exhausted.

The antibody content of the droplet was expressed as the number of lots of bacteria immobilized, and recorded as 1, 1½, 2, 2½, etc. For this purpose, the figure ½ meant partial immobilization of the final lot of bacteria added. The final lot of bacteria was considered to be partially immobilized if 1–3 bacteria remained motile 30 min. after introduction.

Control studies.—In the primary response studies the cell suspension before incubation, the suspending medium and numerous droplets prepared from the cell suspension but containing no cells were routinely tested for freedom from inhibitory activity. In the secondary response studies the suspending medium, single cell droplets before incubation, and the final washing fluid of each cell were similarly tested.

Serum titrations.—Serial two-fold dilutions of the sera from the rats studied were made in physiological saline containing 1 : 100 nutrient broth and 1 : 100 of a 4 hr. broth culture of *Salm. adelaide*. The concentration of organisms in this final suspension was 10^6 per ml. Samples of each serum dilution were transferred on to a glass slide under a layer of paraffin oil, and after 30 min. at room temperature, were surveyed for motility of the organisms. Ninety per cent immobilization was taken as the end-point and the reciprocal of the serum dilution giving 90 per cent immobilization was termed the titre of the serum.

RESULTS

The results of 12 experiments on the primary response are given in Table I and Fig. 1. Out of 662 single cells tested for antibody formation, 15 (2·3 per cent) were found to be active, and in 13 of these the titre of antibody was determined. It was 1 in 8 droplets, 1½ in 4 droplets and 2 in 1 droplet, giving a mean titre of 1·2.

TABLE I.—*Antibody Production in Droplets Containing Various Numbers of Primarily Stimulated Lymph Node Cells*

Experiment	Serum titre	Number of cells in each droplet						Titres‡ of active single-cell droplets
		1	2	3	4	5	6–10	
1	1,600	0*/10†	0/10	1/10	0/5	0/2	4/23	—
2	16,000	1/13	3/18	1/10	1/9	2/18	8/52	N.D.
3	16,000	1/52	2/26	1/18	1/16	2/14	10/56	1½
4	2,400	0/31	0/34	0/17	1/19	2/16	12/63	—
5	1,600	1/25	1/29	1/12	2/21	1/16	10/80	1
6	3,200	0/66	0/46	2/55	1/33	1/31	2/82	—
7	3,000	1/57	2/28	1/26	2/26	0/11	5/57	1
8	2,400	1/61	3/50	3/31	3/24	1/13	15/84	1
9	2,400	5/87	3/86	7/47	4/45	5/33	13/59	1, 1, 1, 1½, 2
10	1,200	0/91	2/65	0/59	0/37	1/19	3/79	—
11	1,600	3/52	3/32	2/19	4/19	2/6	5/24	1, 1½, N.D.
12	2,400	2/117	2/78	2/32	2/26	1/8	14/76	1, 1
Total		15/662	21/498	21/336	21/280	18/187	101/735	(Mean = 1·2)
Percentage of active droplets		2·3	4·2	6·2	7·5	9·6	13·7	

* Numerator indicates number of droplets immobilizing *Salm. adelaide*.
† Denominator indicates number of droplets tested.
‡ Each unit of titre is equivalent to about 10 bacteria immobilized.
N.D. Not determined.

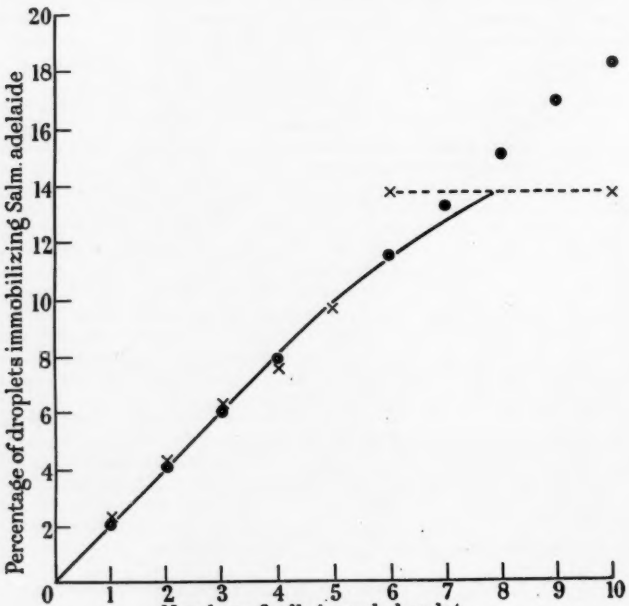

FIG. 1.—Antibody production in the primary response.

× indicates observed value.
×---× indicates observations on droplets containing 6 to 10 cells were pooled.
● indicated theoretical percentage if $n = 0{\cdot}02$ (see text).

Evidence of antibody formation was also sought in 2036 droplets containing from 2 to 10 cells. Fig. 1 shows the percentage of active droplets with various numbers of cells in each droplet. Observations on droplets containing 6–10 cells were pooled. The observed percentages are marked with crosses in Fig. 1. The significance of the dots in Fig. 1 is discussed below.

A further 6 experiments were performed on the secondary response. It was observed that the popliteal lymph nodes in these experiments were somewhat larger than in the previous experiments, and while no accurate estimation of this increase was attempted, the impression was gained that secondarily stimulated

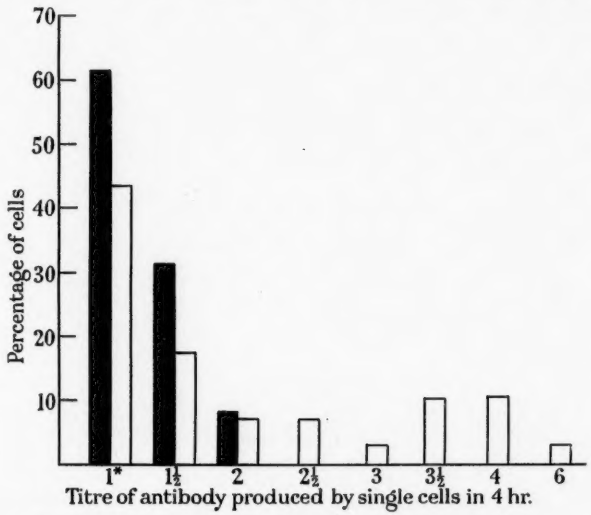

FIG. 2.—The titres of antibody formed *in vitro* by single cells removed during primary or secondary response.
* Each unit corresponds to about 10 bacteria immobilized.
■ indicates primary response.
□ indicates secondary response.

lymph nodes contained 1½–2 times as many cells as primarily stimulated nodes. Single-cell droplets in these experiments were prepared by micromanipulation in order that each cell might be given one individual wash in a large droplet of clean suspending medium. This ensured that the single cell droplets contained no antibody prior to incubation. The results obtained are given in Table II. Of 214 single cells studied, 30 (14 per cent) formed detectable antibody. The titres ranged from 1 to 6, with a mean of 2·0. Fig. 2 compares the titres of antibody formed *in vitro* by cells removed during either primary or secondary response. The difference in the mean antibody titre is statistically significant.

DISCUSSION

It is thought that the most important result of these experiments is the difference in the proportion of cells producing antibody in the primary and the secondary

response. It was observed that the percentage of active single cells was 6 times as high in the secondary response, and if the impression that the secondarily stimulated lymph nodes were at least $1\frac{1}{2}$ times as large as the primarily stimulated nodes was correct, this would mean that there were at least 9 times as many

TABLE II.—*Antibody Production in Droplets Containing Single Secondarily Stimulated Lymph Node Cells*

Experiment	Serum titre	Proportion of active cells	Titres‡ of active droplets
13	32,000	*9/46†	1, 1, 1, 1, 1, $1\frac{1}{2}$, $1\frac{1}{2}$, 2, $3\frac{1}{2}$
14	64,000	6/37	1, 1, 1, $1\frac{1}{2}$, $2\frac{1}{2}$, $3\frac{1}{2}$
15	16,000	1/25	$2\frac{1}{2}$
16	24,000	7/35	1, 1, 1, $1\frac{1}{2}$, 3, $3\frac{1}{2}$, 4
17	12,000	4/46	1, 2, 4, 6
18	4,000	3/25	1, $1\frac{1}{2}$, 4
			(Mean = 2·0)
Total		30/214	
Percentage of active cells		14	

* Numerator indicates number of droplets immobilizing *Salm. adelaide*.
† Denominator indicates number of droplets tested.
‡ Each unit of titre is equivalent to about 10 bacteria immobilized.

antibody-producing cells present in the secondarily stimulated nodes. This difference may be the major factor leading to the higher serum antibody titres obtained at the height of a secondary response. However, these conclusions are open to the possible criticism that a proportion of the cells in the primary response were making amounts of antibody too small to detect by the method used. Study of the results obtained in droplets containing 2–10 cells shows that this is not the case. On the hypothesis that the lymph node consisted of active and inactive cells, and that each active cell produced enough antibody to be detected by the test, the proportion of droplets containing 2 or more cells and showing inhibition would be given by the formula :

$$\text{Proportion} = 1 - (1 - n)^x$$

where n = the incidence of active cells, and x = the number of cells in each droplet. For small values of n and x this relationship is almost linear. In Fig. 1 the observed proportions (expressed as percentages) are plotted for values of x of 2–10. Also illustrated in this figure are the theoretical proportions expected from an incidence of active cells of 0·02 (2 per cent). It will be seen that the line of best fit drawn through the observed proportions coincides almost perfectly with the theoretical values. This seems to show that the method used was sufficiently sensitive to demonstrate antibody production by all the active single cells. A similar conclusion was reached when the hyperimmune response was studied (Nossal, 1958).

A factor contributing to the higher antibody titres observed in the secondary response may be the amount of antibody formed by the active cells in a given time. We observed that in 4 hr. active secondarily stimulated cells produced on the average 1·7 times as much antibody as active primarily stimulated cells. This greater production in a given time *in vitro* may or may not be a true indication of the rate of production *in vivo*.

The findings are in agreement with those of Leduc, Coons and Connolly (1955). In their study of the antibody content of cells by the fluorescent antibody technique, they found that in a secondary response, far more cells fluoresced than in a primary response and they fluoresced more brilliantly, implying greater antibody content.

The above workers (1955) also showed that the majority of cells in a lymph node draining the site of injection of diphtheria toxoid take up antigenic material within 6 hr. Of the current theories of antibody formation (Haurowitz, 1953 ; Pauling, 1940 ; Mudd, 1932 ; Burnet and Fenner, 1949 ; Jerne, 1955 ; Burnet, 1957) only one is directly relevant to the problem of why only a small proportion of these cells should be able to form antibody. This is Burnet's (1957) clonal selection hypothesis, which postulates that there are clones of antibody-producing cells, each clone being predestined to form one sort of antibody globulin molecule. Antigenic stimulation is followed by selective multiplication of representatives of the corresponding antibody-producing clone. It could be postulated that primary antigenic stimulation in our experiments was followed by limited multiplication of cells of the clone corresponding to *Salm. adelaide* flagellar protein, perhaps through 3 or 4 mitotic divisions ; secondary stimulation would then be followed by further multiplication, perhaps through 3 more mitotic divisions, to give a final proportion of 14 per cent of antibody-producing cells.

The author wishes to emphasize that Burnet's hypothesis is still highly speculative. While the above results are consistent with it, they by no means furnish proof. The exact theoretical significance of this work will only become clear when more is understood about the mechanism of antibody synthesis.

SUMMARY

Antibody production *in vitro* by isolated lymph node cells has been studied during the primary and the secondary response.

Rats were injected with isolated flagella prepared from cultures of *Salmonella adelaide* and the draining lymph nodes removed and processed to give single cell suspensions. Microdroplets containing single cells were prepared, and incubated for 4 hr. The amount of antibody formed by the cells was estimated by inoculating motile bacteria into the microdroplet and estimating the number immobilized.

Of 662 single cells tested in the primary response only 15 (2·3 per cent) formed detectable antibody. From the study of 2036 droplets containing 2–10 cells it was concluded that the 97·7 per cent of cells not forming detectable antibody were probably not making any antibody.

Of 214 cells tested in the secondary response, 30 (14 per cent) formed detectable antibody and the average amount formed by each cell was 1·7 times as much as in the primary response.

The theoretical implications of these findings are discussed in relation to Burnet's clonal selection hypothesis.

The author wishes to express his sincere thanks to Sir Macfarlane Burnet and to Professor Joshua Lederberg for their stimulating interest and help.

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PHYSICAL CHANGES IN THE LUNGS DURING RESPIRATORY INFECTION WITH *BRUCELLA SUIS*

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RECENT intensive studies on lung mechanics are not only of physiological interest but also have important applications to the study of diseased lungs. Patients with a wide variety of respiratory diseases have been studied to determine variations from normal in the physical properties of the lungs; there has been, however, an emphasis on chronic pulmonary disorders, and little work has been done on acute respiratory infections. This is to be expected: it is difficult to apply the rather complicated apparatus for investigating pulmonary mechanics to a patient acutely ill and the opportunity for doing so is often short-lived; furthermore the infective process itself may be of greater importance to the patient than any mechanical disadvantage under which his respiratory mechanism may be working.

The only acute pulmonary condition which has been thoroughly studied in this respect is pneumonia, in which early investigators (*e.g.* Siebeck, 1910) showed that the lungs were abnormally stiff. Later measurements (Marshall and Christie, 1954) suggested that this change was not entirely due to loss of tissue through consolidation, but that the ventilated parts of the lungs were also more difficult than normal to distend.

In the course of work on the pathology of pulmonary brucellosis in rabbits we have measured some of the mechanical properties of the lungs before and during infection, and have also assessed the permeability of the pulmonary vascular bed in normal and in infected animals. Although the results are restricted to brucellosis in rabbits, they may bear a general relation to other acute and subacute infections in man, and thus help to fill the gap noted above.

METHODS

Rabbits weighing 2–2·5 kg. were used. Those to be infected were exposed to a passing cloud of single *Brucella suis* organisms, in the apparatus described by Henderson (1952). The exposure time was 1 min., and the cloud concentration from 0·09–1·5 $\times$ 10^6 organisms/l. The rabbits would retain about 9–150 $\times$ 10^3 organisms in the lungs; the lower limit would be ample to infect all the animals, the larger dose is massive.

Mechanical properties of the lungs.—Oesophageal pressure was measured through a saline-filled catheter (1 mm. bore), passed through the mouth and connected to a capacitance-manometer. Intratracheal pressure was measured by a second capacitance-manometer (air-filled). The frequency responses of the manometers with tubes attached were better than 200 cycles/sec. They were used with a cathode-ray oscilloscope and recording camera. The lungs were inflated with a motor-driven syringe which gave linear movement of variable speed and stroke. Volumes up to 100 ml. could be injected at rates of 30–300 ml./sec.

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The experimental procedure was standardized for all rabbits and was the same whether they were infected or not. It was repeated up to three times before exposure to infection, and three times after infection. Control experiments were done on rabbits not exposed to infection, with repeated investigations (up to 6 times) over the same duration of time. An interval of at least 7 days was allowed between experiments, and no series of experiments took more than 2 months for any individual rabbit.

Each rabbit was anaesthetized with pentobarbitone sodium (32 mg./kg. body weight) and immediately curarized (1 mg./kg.), both drugs being given intravenously. An endotracheal cannula was passed through the mouth and artificial ventilation started (30–50 ml., 20 per min., expiration passive); the cannula was tightly wedged in the larynx without leakage of gas. The oesophageal catheter was then passed through the mouth, and the capacitance manometers were connected. A water overflow valve was connected to the expiratory side of the pump and set at 1·5 cm. H_2O; this was to minimize collapse of the lung, since we found that curarization of the rabbits lowered the functional residual capacity, and that this value of expiratory resistance restored the functional residual capacity to the previous "eupnoeic" level.

As soon as a satisfactory record of the two pressures was obtained (2–3 min.) the pump was stopped and the first test inflation with the syringe carried out. Between test inflations the rabbit was ventilated for about 1 min. while resetting the syringe. The order of test inflations was 10, 20, 30, 40, 50 and 60 ml. (sometimes 70 ml. inflations were also done) at a flowrate of 30 ml./sec. The syringe volume was then set at 60 ml. and a series of inflations done at different speeds, 30, 60, 100, 150, 200, 250 and 300 ml./sec. Both series of inflations were then repeated in the same order. The entire procedure took 15–30 min. The rabbit was then kept artificially ventilated until the effect of the curare had worn off, and the cannulae removed and the animal allowed to recover.

Compliance was determined under "static" conditions. One second after the end of a test inflation the intraoesophageal and intratracheal pressures were measured (B, Fig. 1) and the difference gave the transpulmonary pressure at the appropriate volume. The pressures were plotted to give a pressure/volume curve (Fig. 2) which was like that found by Bernstein (1957) who used similar experimental conditions. This method of determining compliance assumes that intraoesophageal pressure is equivalent to intrapleural pressure. In man the 2 are close (Dornhorst and Leathart, 1952); control experiments showed that in rabbits there was usually a maintained pressure difference between the two pressures, but the variations in pressure were similar (Petit and Milic-Emili, 1957). Pressures were therefore expressed relative to the expiratory level of breathing.

Non-elastic resistance ("viscous", mainly airway resistance) was measured from the sudden pressure drop on abrupt termination of the inflation (A, Fig. 1). By recording this value at different speeds of inflation to constant inflation volume a curve relating air-flow to non-elastic pressure drop could be drawn, and from this the non-elastic resistance calculated. Allowance was made for the resistance of the tracheal cannula. The method was similar in principle to the "interruptor technique" of Otis and Proctor (1948).

Stress relaxation was measured as the fall in transpulmonary pressure from the bottom of the non-elastic pressure drop to the point where compliance was determined, 1 sec. later. This was done during rapid inflations, since with slow inflations stress relaxation was hidden in the slow pressure rise. Stress relaxation was not quite complete in 1 sec., but since the transpulmonary pressure declines nearly exponentially after inflation, this was chosen as an arbitrary time of measurement.

Some of the results of physiological interest, especially with regard to stress relaxation, are given elsewhere (Hughes, May and Widdicombe, 1959).

Pulmonary vascular permeability.—This was measured by perfusing rabbits' lungs with blood from a non-pulsatile pump, with low pressure drop (2–4 mm. Hg) between artery and veins. The mean pressure was constant for any series of experiments, and the rate at which oedema formed at that pressure was taken as an index of the permeability of the lung blood vessels. Oedema fluid formation was measured both by changes in the volume of blood in the pump reservoirs, and by weighing the heart and lungs immediately after the perfusion; comparison of the ratio of lung plus oedema fluid to heart weight with normal values of lung/heart ratios (1·53; S.D. = $\pm$0·11) gave the increase in weight due to oedema. Under the experimental conditions oedema formed at approximately linear rates, so knowing the length of perfusion the rate of oedema formation per unit time could be estimated. The 2 methods of estimation of oedema fluid gave comparable results.

This technique has been used to investigate pulmonary fluid dynamics, and is reported in greater detail elsewhere (Hughes, May and Widdicombe, 1958*a*).

Lung blood contents were measured by the method of Ponder (1942) slightly modified. This method involves maceration and digestion of the lung, and the colorimetric estimation of its iron content.

Bacterial cultures.—Lungs, other organs and blood were tested for living *Br. suis* organisms by plating and incubation on fortified tryptose agar (containing 1 in 8 $\times$ 10^6 Brilliant Green and 4 units/ml. polymyxin (Burroughs Wellcome) to prevent overgrowth with contaminants).

RESULTS

Mechanical Properties of the Lungs

Twelve rabbits were investigated before and up to thirty days after exposure to *Br. suis*. The cloud concentration was 0·09 $\times$ 10^6 organisms/l. (estimated retention 9 $\times$ 10^3 organisms). Over the eupnoeic range of volumes (0–30 ml.,

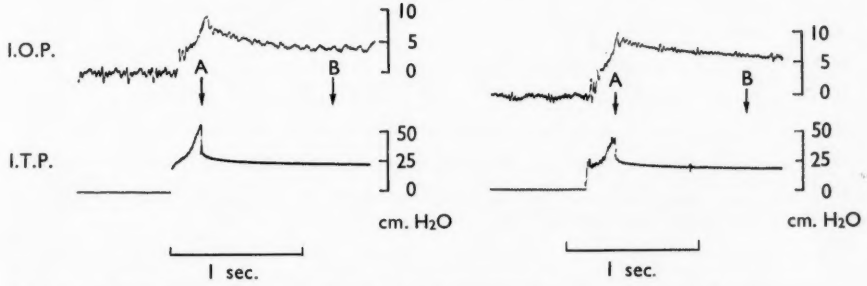

FIG. 1.—Rabbit, 2·2 kg. Record of oesophageal pressure (above) and intratracheal pressure (below) during inflations with 60 ml. air, at 300 ml./sec. A—termination of inflation. B—1 sec. later, at which point compliance and stress relaxation were determined. The record on the left was taken 4 days before exposure to infection; that on the right was obtained 14 days after infection. A fall in stress relaxation and an increase in static compliance can be seen. The pressure fall due to non-elastic resistance is decreased.

transpulmonary pressures up to 8 cm. H_2O) there was no constant change in the pressure/volume curve of compliance after infection, and in any individual the alteration was within the limits of variation seen on repeated measurements before infection. With large inflations (30–70 ml., transpulmonary pressures of 10–25 cm. H_2O), however, there was usually an increase in compliance (*i.e.* the lungs were less stiff). This was seen in 10 of the 12 rabbits. Fig. 1 is an example of records obtained before and after infection. Fig. 2 shows pressure/volume curves from the same rabbit. Fig. 3 gives the results (mean and S.E.M.) from all of the 12 animals. With large volumes there was usually a decrease in stress relaxation, corresponding to the increase in compliance; the transpulmonary pressure was lower throughout its entire course after the end of inflation (Fig. 4).

There were less consistent changes in non-elastic resistance. Four rabbits showed an increase in this, and 3 a decrease, the remaining 5 showing no significant change. This quantity is, however, very variable in normal rabbits on repeated investigation (possibly owing to the action of curare), and the changes found in

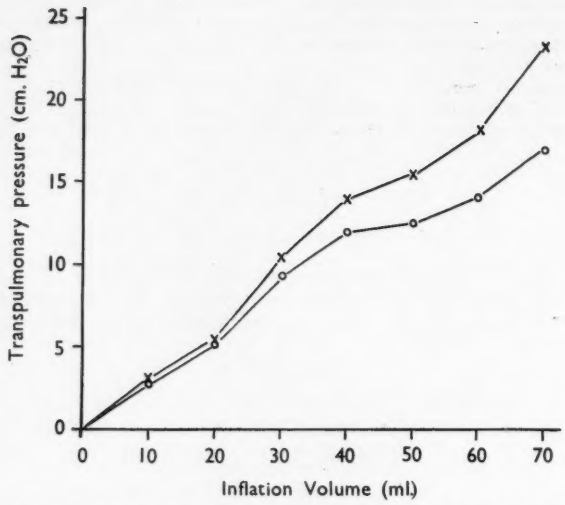

FIG. 2.—Same rabbit as in Fig. 1. Pressure/volume curves obtained at the same times as the 2 records in Fig. 1. ×——× before infection. ○——○ after infection. There is an increase in compliance with large volumes. For clarity only 2 curves are drawn, although 12, on 6 occasions, were obtained for this rabbit.

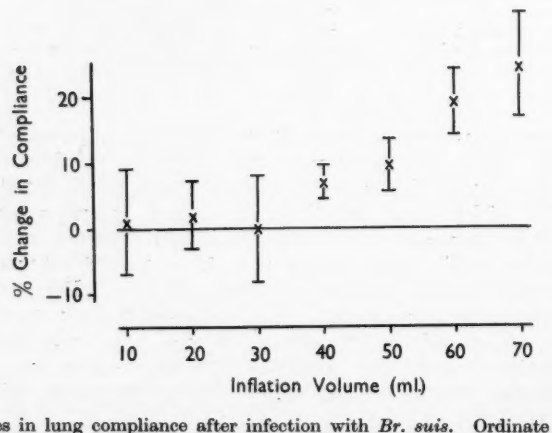

FIG. 3.—Changes in lung compliance after infection with *Br. suis*. Ordinate: percentage change in compliance. Abscissa: inflation volume. The means with standard errors are given for 12 rabbits (7 rabbits for the 70 ml. inflations), on each of which several measurements were made before and during infection. The horizontal line corresponding to zero per cent (ordinate) represents the means of estimations before infection.

brucellosis were certainly within the limits of this variation. It is chiefly an estimate of airway resistance, and since pulmonary brucellosis may affect the bronchi changes in this were anticipated but not consistently found.

Pulmonary Capillary Permeability

Eight rabbits were exposed for 1 min. to a cloud containing $1{\cdot}50 \times 10^6$ organisms/l., and the lungs perfused at a mean perfusion pressure of 30 mm. Hg. The mean rate of oedema fluid formation was $24{\cdot}6 \times 10^{-4}$ ml./sec./g. lung (S.E.M. = 3·25). By comparison 10 control rabbits gave a mean value of $14{\cdot}1 \times 10^{-4}$ ml./sec./g. lung (S.E.M. = 2·02). The difference between these 2 means is statistically significant ($P < 0{\cdot}01$). The experiments were done from 4–30 days after exposure to infection, and there was no correlation between the change

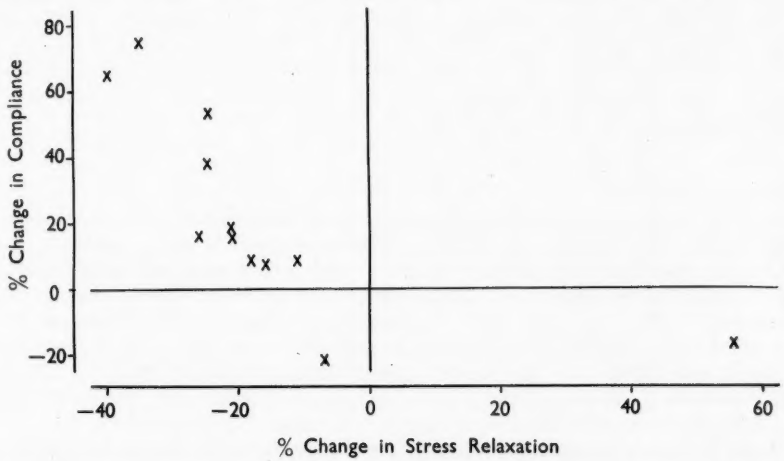

FIG. 4.—The correlation between the percentage change in compliance (ordinate) and the percentage change in stress relaxation (abscissa). Each point is for the maximal volume of inflation (60 or 70 ml.) for one animal, based on the mean of estimations before infection and the mean of estimations after infection. The one very divergent point was from a rabbit thought to have developed pneumonia.

in permeability and the time from infection. In these experiments we measured the blood content of the lungs after perfusion and found it considerably increased compared with normal rabbits. This partly accounts for the greater increase in lung weight in infected rabbits on perfusion, but if allowance is made for this congestion (by subtracting the total weight of lung blood from the increase in weight on perfusion) there is still a significant difference between the rabbits with brucellosis and the controls (means : infected rabbits, $18{\cdot}9 \times 10^{-4}$ ml./sec./g. lung, S.E.M. = 3·72 ; controls, $10{\cdot}5 \times 10^{-4}$ ml./sec./g. lung, S.E.M. = 1·50 ; $P < 0{\cdot}01$).

A second series of rabbits was exposed to a smaller dose of organisms (cloud concentration $0{\cdot}09 \times 10^6$ organisms/l.). On the 5 rabbits tested only 2 had

pulmonary capillary permeabilities well above the normal range and these were the only 2 which gave positive cultures from the lungs; the other 3 also had brucellosis as judged by post mortem and body temperature changes.

Histological Changes

Previous studies of the microscopic pathology of pulmonary brucellosis showed that in guinea-pigs there are no detectable changes in the alveoli although lesions in the bronchi and lymphoid tissue may be striking (Elberg and Henderson, 1948). The histological reports on the lungs of the rabbits in our experiments stated that there was some interstitial thickening of the alveolar walls with an increase in mononuclear cells, some of them containing organisms; these appearances were infrequent and slight, however, and were possibly influenced by the degree of collapse or expansion of the lung. It is possible that there is a species difference between rabbits and guinea-pigs. The changes in the bronchial walls were more specific, there being small scattered granulomata, with many large mononuclear cells. In animals killed several weeks after exposure there were also necrotic nodules, especially in the bronchial walls. The lymph nodes showed changes typical of brucellosis.

DISCUSSION

In spite of pathological changes in the lungs, the static compliance was not altered over the eupnoeic range, and was increased over a range probably corresponding to the rabbits' inspiratory reserve. This was a surprising result, especially in view of Marshall and Christie's (1954) observations on patients with pneumonia. These patients had decreased compliance, often less than 50 per cent of normal, and the authors concluded that this was not entirely due to consolidation of the lung, but that apparently normal areas of the lung were more rigid than usual. No comparable observations on humans with non-consolidating lung infections are known.

Since the compliance of the lungs is not greatly affected by changes in pulmonary capillary pressure both in dogs (Borst, Berglund, Whittenberger, Mead, McGregor and Collier, 1957) and in rabbits and cats (Hughes, May and Widdicombe, 1958*b*), nor probably by bronchial tone (Radford and Lefcoe, 1955), it would not be very surprising if a diffuse lung infection caused little increase in rigidity; but the observed increase in compliance is both unexpected and difficult to explain. Compliance depends primarily on the arrangement of elastic and reticular fibres, on surface tension effects in the alveoli (Radford, 1957) and on the numbers of patent and collapsed alveoli (Bernstein, 1957); it is reasonable to suppose that one of these factors is altered.

The most likely possibility was thought to be alveolar collapse with overdistension and increased compliance of adjacent alveoli; but not only is there no histological evidence for this, but it would exert most effect at low lung volumes (where compliance was unchanged) rather than at large volumes (where full opening of the alveoli is probably present in unconsolidated lungs). It should be pointed out that the lower part of the pressure/volume curves, which was not affected by infection, acts as a control for the upper range; there is thus a strong indication that the increased distensibility is not due to

collapsed alveoli. Clements, Brown and Johnson (1958) have indicated the means whereby the surface lining of the alveoli determines compliance and lung hysteresis ; if stress relaxation is due to the same mechanism as hysteresis (Hughes *et al.*, 1959) then the fact that infection with *Br. suis* reduces both stress relaxation and lung stiffness could be an indication that the alveolar surface behaviour is changed. But if this were so one would expect an alteration in compliance at low volumes, too, where surface tension effects are also important. There is thus some reason for rejecting the possibilities of alveolar collapse and of changed surface activity, and the third possibility (that the structural elements in the alveoli are deranged or altered) is considered rather more likely.

A change in alveolar compliance may also account for the results on capillary permeability. For if the alveoli are more distensible on inflation with large air volumes, the vascular bed may be more distensible on inflation at high blood pressures. The fact that heavily infected lungs not only produced oedema fluid more rapidly but contained a higher volume of blood than the controls can be cited to support this ; but it should be pointed out that the blood volumes were measured in lungs only after oedema formation, and that the increase in blood content could be due to extravasation as well as to engorgement.

The increase in pulmonary capillary permeability was seen only after exposure to large numbers of organisms (or when lung culture plates were strongly positive) and is presumably associated with these organisms in the lung parenchyma. This view is supported by experiments in which the lungs of five normal rabbits were perfused with blood from donors with brucellosis ; there was no increase in permeability. This last experiment is not, of course, conclusive, since infected blood might contain substances which increase capillary permeability but take a relatively long time to act, but it suggests that it is the pulmonary infection and not the infected blood which is responsible ; and infected rabbits with only a scanty growth of organisms on plating the lungs did not show any increase in permeability, even if the blood contained organisms. Whether the change in permeability is due to the action of a bacterial toxin in dilating pulmonary capillaries or in damaging their walls or a combination of both is not known. In pulmonary brucellosis there is no alveolar oedema, although it is not clear to what extent the alveolar thickening is associated with interstitial oedema. Harper (1955) has shown that in guinea-pigs *Br. suis* can multiply readily and rapidly in the lung before lymph node and general invasion, and he suggests that this growth occurs in the alveoli. The site and timing of this bacterial multiplication fits well with the physiological changes found in our experiments.

Pulmonary brucellosis in man is not common, and our results can only be applied to human lung infections in a negative sense ; even with diffuse thickening of the alveolar wall there need not necessarily be increased rigidity of the lungs, and generalized extension of micro-organisms through the lungs can cause increased capillary permeability without pathological evidence of oedema. It is reasonable to suppose that there would be an increased liability to pulmonary oedema in those circumstances.

A second, more speculative application is offered. It has long been known that the mechanical changes in the lungs in diffuse hypertrophic emphysema are usually preceded by chronic respiratory infections such as bronchitis (Laennec, 1829 ; Leopold and Gough, 1957). Search for an aetiological connection between the two conditions has led to many, often obscure hypotheses, most of which

include damage to elastic tissue in the alveoli and an increase in compliance with over-distension. *Br. suis* in rabbits causes greater distensibility of the lungs; if the more common organisms found in chronic bronchitis have a similar action this might be a factor in the production of emphysematous changes.

SUMMARY

Rabbits have been infected with *Brucella suis*, inhaled into the lungs as single organisms, and lung physiology investigated in an attempt to correlate it with pathological findings.

In spite of some thickening and cellular infiltration of the alveoli there was no evidence of increased rigidity of the lungs in the eupnoeic range of inflation volumes, and a decrease in rigidity was found above this range.

Pulmonary capillary permeability was investigated in perfused lungs; with pulmonary brucellosis there was a considerable increase in permeability as measured by the rate of formation of oedema fluid.

Possible applications of these results to infections of the lungs in man are discussed.

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A STUDY ON SEROTHERAPY OF POLIOMYELITIS IN RHESUS MONKEYS

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In work previously reported (Liu, Carter, DeSanctis, Geating and Hampil, 1958) a severe infection in the central nervous system (CNS) of monkeys was induced by the intraspinal inoculation of a small amount of the Mahoney strain of Type I *poliovirus hominis*. Paralysis of the infected animals could be prevented or limited by the administration of rabbit antiserum. In the majority of the experiments the serum was administered when the infected monkeys showed no apparent clinical symptoms; such management, strictly speaking, should be considered as prophylaxis. However, encouraging results were obtained with small groups of animals that received delayed treatment. Although only 8 monkeys were employed in the experiment in which the serum was injected at 48 hr. post-infection, the authors concluded tentatively that the therapy was effective in preventing further spread of the infection in the CNS.

In view of these favorable results, further work was carried out. In the present study, emphasis was placed on the therapeutic aspect of poliomyelitis, dealing mainly with the following questions: What happens when delayed treatment is applied to more animals? What are the clinical signs of the infected monkeys at the time of therapy? What is the effect of serotherapy if infection by another route is employed? What is the effect of antibody obtained from human sources in treating infected monkeys?

MATERIALS AND METHODS

Virus used for infection of monkeys

The Mahoney strain of Type I *poliovirus hominis* was employed throughout the experiments for infection of the monkeys. The procedures for preparation of the seed virus, production of the monovalent vaccine and titration of the virus intraspinally in rhesus monkeys have been described (Liu *et al.*, 1958). In addition to the intraspinal injection of virus, in some experiments intracerebral inoculation was used. The procedures of inoculation *via* this route were described in detail by Bodian, Morgan and Schwerdt (1950). All animals were anesthetized by injection of 30 mg./kg. of sodium pentobarbital intravenously. Under deep anesthesia, each animal was infected with 500,000 50 per cent tissue culture infective doses ($TCID_{50}$) of Mahoney virus in 1 ml. of medium 199 (Salk, Youngner and Ward, 1954) by injecting one-half the volume into each of both thalami through a hole in the skull made by a small "Moto tool" over the central parietal region, about one-half inch lateral to the sagittal suture.

Hyperimmune rabbit serum

The method for preparing the Type I poliovirus hyperimmune serum in albino rabbits has been described in the previous report (Liu *et al.*, 1958).

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Hyperimmune human γ-globulin

A pool of plasma was obtained from 39 donors as described in a later section. The plasma was fractionated according to a combination of methods: No. 6 (Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor, 1946) and No. 9 (Oncley, Melin, Richert, Cameron and Gross, 1949). The distribution of plasma proteins by electrophoresis has been described by Cohn *et al.* (1946) and Oncley *et al.* (1949).

Titrations of $TCID_{50}$ and neutralizing Antibodies

The colorimetric method of Salk, Youngner and Ward (1954) was adopted, with a minor modification that 2 per cent calf serum was substituted for the horse serum in mixture 199. Virus dilutions were set up at 0·5 and 1·0 log steps and antibody dilutions in 2-fold steps. The 50 per cent end point of these titrations was calculated according to the method of Reed and Muench (1938).

The serum was tested for neutralizing antibodies against not only Type I *poliovirus hominis* but also the MEF_1 (Type II) and Saukett (Type III) strains originally obtained from Dr. J. E. Salk and designated M-80 and M-83 respectively. The stock cultures were used at their third passage in monkey kidney cells. The $TCID_{50}$ lay between $10^{-5\cdot3}$ and $10^{-7\cdot3}$ ml.

RESULTS

Studies with hyperimmune rabbit serum

Delayed treatment after intraspinal inoculation. Two experiments were carried out in order to ascertain the efficacy of the delayed therapy with the hyperimmune serum prepared from the rabbits and to determine more fully the clinical signs at the time when therapy was given. Each experiment included 12 rhesus monkeys weighing 2–3 kg. each. In the first trial, 8 animals received an intravenous dose of 20 ml./kg. of the standard hyperimmune rabbit serum intravenously as described in the previous report (Liu *et al.*, 1958), and 4, which received no serum, were controls. In the second trial, 7 were treated and 5 used as controls. All monkeys were infected intraspinally with 10–16 $TCID_{50}$ of Mahoney virus 48 hr. prior to therapy. Thereafter, these animals were under careful observation for a period of 4–5 weeks.

Two objective criteria were applied to establish the clinical diagnosis of poliomyelitis in the infected monkeys when therapy was administered: elevation of body temperature and presence of muscular paralysis. The clinical signs of each monkey in these 2 experiments at 48 hr. post-infection are shown in Fig. 1. It is known that the rectal temperature of normal monkeys varies a great deal. Nonetheless, it is reasonably certain that a temperature above 39·5° may be considered abnormal. As is shown in the figure, approximately half the animals had developed pyrexia at the time therapy was started. Also, as indicated by the blackened areas in the monkeys, all manifested a certain degree of paralysis, involving from one of the lower legs to both of them. In our experience, any grade of paralysis beyond paresis of one of the lower legs is unequivocally due to the viral infection.

The fate of these animals is summarized in Fig. 2. As can be observed, 3 of 4 and 4 of 5 in the control groups of the first and second experiments, respectively, died from severe paralysis caused by the viral infection. Thus the total mortality was 7 of 9 or 78 per cent. The muscular paralyses of the monkeys that succumbed to the infection extended to the entire body and 4 limbs, except for 2 animals (16 in exp. 10, and 16 in exp. 33); these animals died too soon to permit full development of paralysis. Such a phenomenon has been described previously (Liu *et al.*, 1958) and may be ascribed either to an extensive encephalitis or to rapid involvement of the majority of the respiratory muscles. Two monkeys which survived for the entire period of observation displayed paralysis of muscles below the waistline. In addition to the paralysis of the lower body and limbs, one of the animals (15 in exp. 33) showed paralysis of the left facial muscles, a condition that occurred rarely after an intraspinal inoculation.

In the treated groups, only one monkey (12 in exp. 33) succumbed to the infection with complete paralysis on the 5–6th day post-infection. The reason for this one failure remains obscure, because no necropsy could be performed. Two additional animals (17 and 22) in exp. 10) expired in the 4th week post-infection from uncontrollable diarrhoea, but their paralyses were definitely less extensive than those of controls. Therefore, the serum treatment

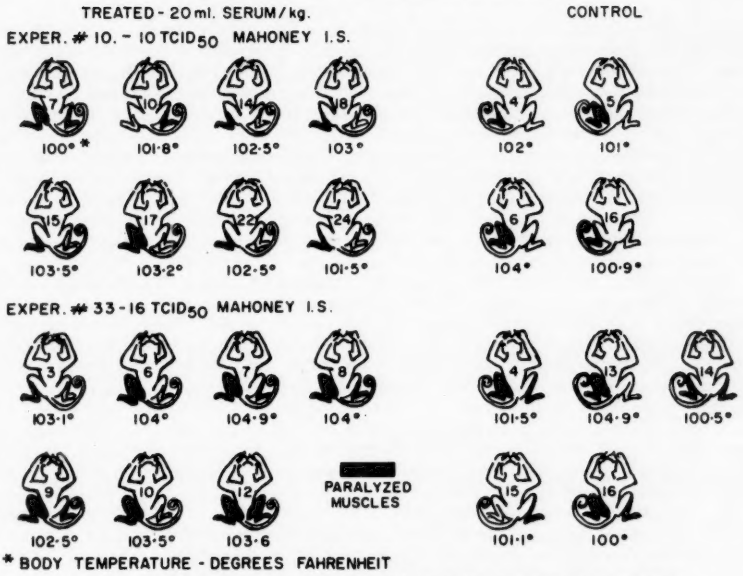


FIG. 1.—Clinical signs in monkeys at 48 hr. after intraspinal inoculation of 10–16 $TCID_{50}$ of Mahoney strain of poliovirus.

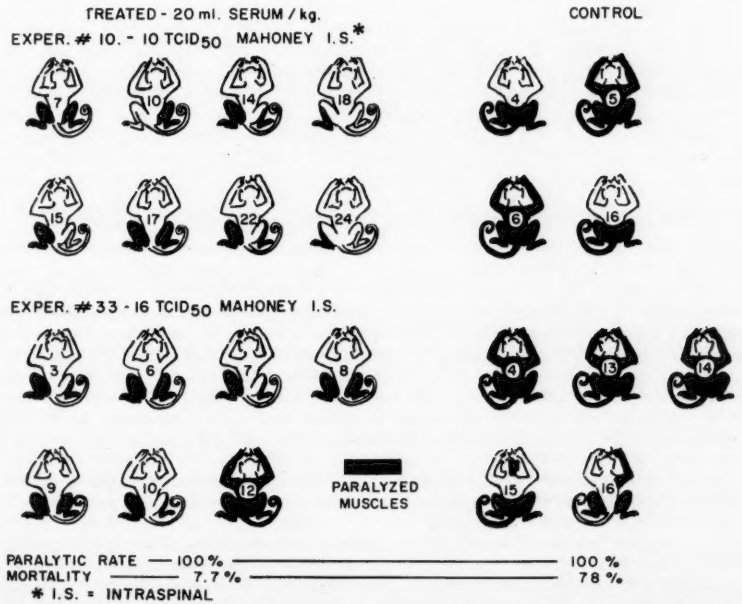


FIG. 2.—The fate of the monkeys shown in Fig. 1 ($P < 0{\cdot}01$; 95 per cent confident limits of mortality ; treated 0·2–32 per cent and controls 40–97 per cent).

for these 2 monkeys should be considered successful. The remaining monkeys survived with limited paralysis for the entire follow-up period. The mortality of the treated groups thus averaged 7 per cent (1 of 15). Even if the other 2 animals that died from diarrhoea were assumed to have succumbed to poliomyelitis, the total mortality would then be only 20 per cent which still would be substantially lower than that found in the control groups (78 per cent). With regard to the degree of muscular paralysis of the treated animals, it should be emphasized that at the termination of observation no monkey acquired a paralysis involving more than the 2 lower limbs, except 12 of exp. 33, listed above. Hence, there appeared no doubt that the extent of muscular disability in the treated was much less than that in the controls.

Comparison of Figs. 1 and 2 shows that the progression of muscular paralysis in the treated animals was completely arrested within a period of 1–3 days after administration of the serum. The length of time required varied in individual monkeys. The possible reasons for such a delay in stopping the progress of the disease are discussed in a later section.

Serotherapy of poliomyelitis in monkeys infected intracerebrally. Only intraspinal infection was used throughout the prior study. One may wonder what would be the efficacy of the treatment if infection through another route were employed. Among the possible routes for infection, intrathalamic inoculation was selected for further study.

The first experiment included a total of 11 monkeys. Each animal was infected intracerebrally with 500,000 $TCID_{50}$ of Mahoney virus in 1 ml. of medium 199. The animals were examined daily for rectal temperature, muscular weakness and other neurologic signs. On the basis of clinical observation, 2 animals which appeared conspicuously sick were selected for treatment on each of the following days: 3rd, 5th, and 7th day post-infection. The treatment consisted of an intravenous injection of 15 ml./kg. of the hyperimmune rabbit serum. The remaining 5 monkeys, untreated, served as controls. Among these 5, one monkey was sacrificed on the 3rd and another on the 7th day post-infection. The viral contents of various levels of the CNS were examined according to the method described in the previous report (Liu *et al.*, 1958). It was found that as early as the 3rd day after inoculation the virus was disseminated throughout the CNS except in the occipital lobe, and significant quantities, $10^{3.6}$ to $10^{6.0}$ $TCID_{50}$ per g. of tissue, were found at all levels in the spinal cords of these 2 sacrificed animals.

In the second experiment, 6 monkeys were employed. Two groups of 2 monkeys received 5 ml./kg. and 10 ml./kg. of the same serum, respectively on the 6th day after infection with the identical amount of virus. The remaining 2 animals without treatment were controls.

All animals in these experiments were observed for 5 weeks after the infection. The results revealed that all the 6 monkeys treated with 15 ml./kg. were free of any detectable muscular paralysis at the end of the observation period, whereas the 5 controls and the 4 monkeys receiving less than 15 ml./kg. of serum showed varying degrees of paralysis involving 2–4 limbs.

The clinical course of poliomyelitis in monkeys following intracerebral inoculation

The clinical course of the disease in monkeys after intrathalamic infection differed considerably from that of intraspinal inoculation. Typical clinical records of an untreated monkey and monkeys that received the serum 3, 5, and 7 days post-infection, respectively, are shown in Fig. 3. In general, on the first day after inoculation, the untreated animals invariably showed elevated rectal temperatures which lasted for only 1–2 days. The fever began to fall by lysis on the following day and the temperature stayed within normal range for the next 1–4 days. During this period, the animals appeared alert as usual. Thereafter, the second spike of temperature developed, and the animals manifested anorexia, sluggishness roughened coats and other neurologic signs *i.e.*, tremor, unsteady gestures, irritability. As the lysis of the second spike began, weakness of one of the limbs, ptosis of the eyelid, or deviated neck became detectable. The paralysis progressed for a period of 1–2 weeks. Finally, a wide involvement of muscles of limbs, face and neck resulted. In general, the paralysis was not severe enough to cause death and the majority of the animals lived until the end of the usual observation period.

As shown in the top chart of Fig. 3, a typical picture of an untreated control (12) is illustrated. Although the monkey showed involvement of 4 limbs at the end of 2 weeks, sufficient movements of its neck and hands persisted permitting the animal to live until the end of the experiment. In the second chart, monkey 6 was treated on the 3rd day post-

infection when the second elevation of temperature was apparent. Two days after treatment, the monkey appeared sick. This lasted for only one day, and throughout the rest of the time the animal was in good health. The third chart shows monkey 1 that received the serum on the 5th day following infection. At that time, pyrexia and shaking of the head were observed. The latter sign persisted for 6 days. In addition, paresis of the left lower leg was noticed between the 7th and 9th day. However, the animal recovered completely by the end of the second week and stayed well for the rest of the period. In the last chart, monkey 7 was treated on the 7th day post-infection when fever had been present for 3 days and at the time of treatment neurologic signs were evident. Despite these, the monkey did not develop permanent paralysis and appeared normal again on the 3rd day post-treatment.

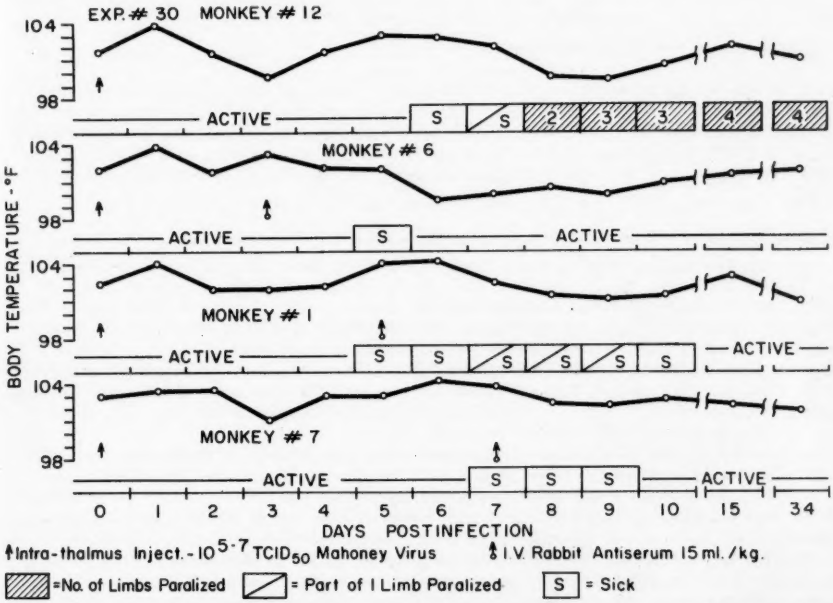


FIG. 3.—The effect of serotherapy on polio infection in Rhesus monkeys after intracerebral inoculation of 500,000 $TCID_{50}$ Mahoney strain of poliovirus.

Antibody levels in the blood of monkeys before and after treatment

In all experiments which were carried out, including those in the previous paper (Liu *et al.*, 1958), serum specimens were taken from the monkeys routinely before and after the infection and their neutralizing antibodies against Mahoney virus were titrated. Since many reports have appeared in the literature concerning this subject, these data have not been presented. However, in order to illustrate the antibody content in the blood of animals before and after treatment with the immune serum, the results of the titrations of the sera obtained from 4 animals in the first experiment using intracerebral challenge are summarized in Table I.

As can be seen, no animal had demonstrable antibody in the serum before treatment. At 10 min. after the treatment was given, the neutralizing antibody against the Mahoney virus in the serum increased to an enormous level. The arithmetic mean titre of the 4 specimens was 17,700 units per 0·5 ml. In the experiment, the animals received 15 ml./kg. of rabbit serum with 75,000 units of antibody per 0·5 ml. Therefore, a total of 2,250,000 (75,000 × 15 × 2) units of antibody should be present in every kg. of body weight of these animals. If approximately 10 per cent of the body were whole blood, the volume of plasma would be

TABLE I.—*Neutralizing Antibody Titres against Mahoney Strain of Poliovirus in the Blood of Monkeys Before and After Treatment with Hyperimmune Rabbit Serum*

Monkey No.	Serum taken: 10 min. before treatment	Serum taken: 10 min. after treatment
1	<5*	15,600
6	<5	16,900
8	<5	17,000
11	<5	21,400
Average	<5	17,725

* Reciprocal of the 50 per cent neutralizing end point.

about 50 ml. per kg. of body weight. Theoretically, the total amount of antibody should be distributed evenly into 65 ml. of the intravascular fluid (50 ml. of plasma and 15 ml. of the injected serum), hence the titre of the serum of the animals immediately after treatment should be 1 : 17,300 per 0·5 ml. The average titre of the neutralizing antibody found in the animal serum, 1 : 17,700, is indeed quite close to the theoretical calculated value recorded above.

Preparation of hyperimmune anti-polio γ-globulin from human plasma

Seventy-five donors were selected from the Philadelphia Donor Center of Merck Sharp and Dohme Division, Merck and Co., Inc. Each was immunized twice with 1 ml. of Salk vaccine intramuscularly at an interval of 2 months. Samples of serum were procured from all donors prior to immunization. Titres of neutralizing antibodies against the 3 types of *poliovirus hominis* were determined by Microbiological Associates, Inc. It was found that all pre-immunization sera of the 39 donors who had completed the course of 2 injections contained antibodies against at least one type of virus ; 33·4 per cent had antibodies against any combination of 2 types and 61·5 per cent, antibodies against all 3 types. The titres varied from 1 : 4 to greater than 1 : 1024.

One pint of blood was collected from each of these 39 donors between 1 and 2 weeks after the second injection. The plasma samples from these bleedings were pooled and fractionated according to the methods indicated previously. The pool of plasma and the 16 per cent solution of γ-globulin prepared from it were tested repeatedly for neutralizing antibodies against 3 types of poliovirus. The average titres from 4 different assays for the plasma and γ-globulin and the arithmetic mean titres of the 39 sera obtained before immunization are shown in Table II. It is evident that the levels of antibody in the blood of these donors increased from 77- to 154-fold after a course of 2 injections of vaccine. An approximate 8-fold concentration was achieved in the 16 per cent γ-globulin as compared with the original plasma pool.

TABLE II.—*Titres of Neutralizing Antibodies against 3 types of Poliovirus of the Sera from 39 donors before immunization, of the hyperimmune plasma pool and of γ-Globulin prepared from the pool*

	Reciprocal of the 50 per cent neutralizing end point: Type I (Mahoney)	Type II (MEF_1)	Type III (Saukett)
Prevaccination serum	126	87	76
Post vaccination plasma	9,700	13,400	6,000
Folds increased (compared with prevaccination)	77	154	80
γ-globulin 16 per cent solution	75,050	86,280	52,130
Concentration factor (compared with plasma)	7·7×	6·4×	8·7×

Samples were removed from each step during the process of fractionation. Levels of antibody against 3 types of poliovirus were determined for all the samples, and the final

preparation, 4·7 per cent γ-globulin. The total units of antibody in each fraction were computed by multiplying the titre by its corresponding volume in litres. The percentage of the antibody content in every preparation was calculated on the basis that the number of units in the pool of plasma is considered as 100 per cent of the source of the antibody. These results are summarized in Table III.

TABLE III.—*Neutralizing Antibodies against 3 types of Poliovirus in Various Fractions of Human Hyperimmune Plasma*

Fraction	Amount in litres	Type I antibody			Type II antibody			Type III antibody		
			Total units			Total units			Total units	
(per cent)		Titre	(×10³)	per cent	Titre	(×10³)	per cent	Titre	(×10³)	per cent)
Plasma (5·2)	14·2	8,050*	114·31	100·0	12,800	181·76	100·0	9,000	127·80	100·0
Fraction I (3)	1·7	154	0·26	0·23	600	1·02	0·39	400	0·68	0·53
Sup I (2·35)	31·8	4,160	132·20	115·65		N.D.		4,460	141·85	110·99
Sup. II & III (4·26)	12·0	524	6·29	5·50	1,048	13·58	7·42	1,230	14·76	11·55
Sup. II & IIIw (0·46)	10·0	1,200	12·00	10·50	800	8·00	4·40	800	8·00	6·03
Sup. II	25·0	63	1.58	1·39	100	2·50	1·38	143	3·58	2·72
Ppt. III (2·5)	2·5	2,240	5·60	4·90	2,240	5·60	3·08	2,240	5·60	4·38
γ-globulin (4·7)	1·5	30,800	46·20	40·42	38,400	57·60	31·69	30,800	46·20	36·15

* Reciprocal of highest dilution neutralizing 10–50 $TCID_{50}$ of 3 types of *poliovirus hominis* (50 per cent end point).
N.D. = Not done.

As is revealed, a considerable quantity of neutralizing antibody was lost in various fractions and in various steps during processing. In the final product, γ-globulin contained only 31–40 per cent of the total antibody present in the plasma. Fraction I (fibrinogen, antihaemophilic globulin, etc.) contained 0·23–0·53 per cent of the entire antibody; supernatant II + III (albumin, α-lipoprotein, metal binding globulin, etc.) 5·5–11·5 per cent; supernatant II + III-W (lipoprotein and a small quantity of γ-globulin), 6·0–10·5 per cent; supernatant II (residual γ-globulin), 1·4–2·7 per cent and precipitate III (iso-agglutinin, prothrombin, plasminogen, fibrinogen, γ-globulin, etc.) greater than 3·1–4·9 per cent. Presumably, the latter fraction should contain considerably more antibody than was shown (Sanders, unpublished). Unfortunately, this particular test cannot be repeated because of a limited supply of certain samples. Also, supernatant III containing the γ-globulin fraction should have been assayed prior to fractionation and precipitation. At any rate, there was approximately 30 per cent of antibody which cannot be accounted for. This loss may be ascribed to the filtration and other manipulations during the process. However, there is no way to ascertain whether or not inactivation or denaturation of the antibodies had occurred.

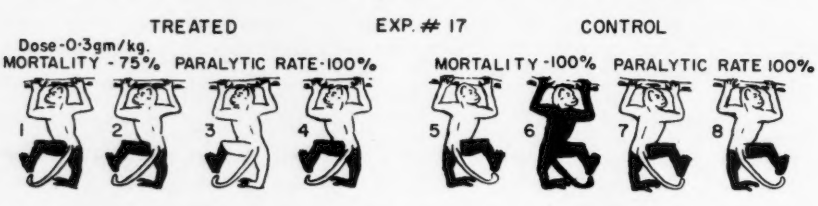
Treatment of poliomyelitis in monkeys with human γ-globulin

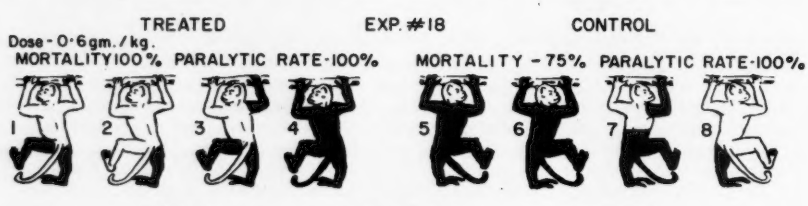
Three experiments were carried out to test the efficacy of the prepared γ-globulin in treating poliomyelitis in monkeys. Six to 8 rhesus monkeys weighing 2–3 kg. were used for each experiment. Immediately after intraspinal infection with 100 $TCID_{50}$ of Mahoney virus, half the animals were treated with the 10–16 per cent aqueous solution of human γ-globulin intravenously and the remainder used as controls. The amounts of γ-globulin employed in these experiments were 0·3, 0·6 and 1·5 g/kg., respectively. All animals were observed for a period of not less than 4 weeks following infection and therapy. Special attention was paid to their muscular activities for the entire period. The outcome of these experiments are illustrated in Fig. 4. As is shown in the figure, almost half the monkeys died too rapidly to develop an extensive paralysis, probably for the reasons which were referred to previously, *i.e.*, disseminated damage in brain stem or paralysis of the respiratory muscles. The number of animals developing such fulminating disease increased as a larger infectious dose was employed.

In the groups treated with 0·3 and 0·6 g./kg. of γ-globulin, there was no difference in the course of illness and the extensiveness of muscular involvement as compared with the corresponding controls. In contrast, definite therapeutic effects were observed in the animals receiving 1·5 g./kg. of the human material. None of the treated group died while all the

controls succumbed to the infection. One monkey (2) among the treated died on the 17th day post-infection from apparent enteritis and malnutrition. This same animal showed a partial paralysis and the other 2 appeared to have no disability, whereas all the controls were completely paralyzed. From these data, it appeared that the antibodies derived from human sources were as effective in arresting polio infection in the CNS of monkeys as those of rabbit origin.

It has been reported (Stokes, 1944 and Janeway, personal communication) that shock can be elicited in humans by giving γ-globulin intravenously. Shock occurred in the above experiments when the hyperimmune human γ-globulin was injected into monkeys. Generally, administration of the total volume of the globulin took 10–15 min. for each animal. About





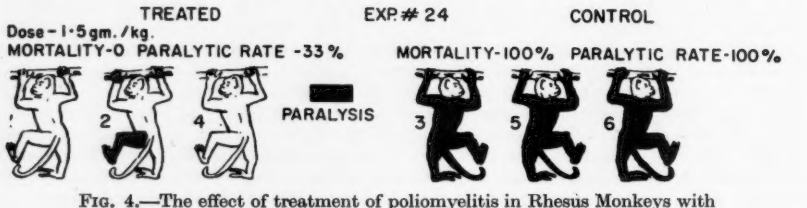


FIG. 4.—The effect of treatment of poliomyelitis in Rhesus Monkeys with varying amounts of hyperimmune Human γ-globulin.

25 per cent of the treated animals developed shock-like symptoms at the early stage of injection, within 1–2 min. after beginning the injection. It seemed that this adverse reaction did not appear to be related to the volume and concentration of the preparation used, but rather that certain animals were apparently sensitive to this material. Fortunately, no monkey succumbed from this reaction which was controlled by subcutaneous injection of 0·1–0·2 ml. of 1 : 1000 epinephrine either prior to administration of the γ-globulin or immediately following the appearance of early signs of this syndrome, *i.e.*, increased depth of respiration and pallor of face.

DISCUSSION

As early as 1944, Stokes analyzed the possibility of serotherapy of viral infections and indicated its future potential. In the following decade, various investigators attempted to treat a score of viral diseases with specific antiserum or its concentrates. Some encouraging information was obtained for measles (Stokes, Maris and Gellis, 1944); mumps (Derman and Lehew, 1944; Gellis,

McGuinness and Peters, 1945); rabies (Habel, 1945; Ghodssi, 1947, and Koprowski and Cox, 1951); equine encephalomyelitis (Zichis and Shaughnessy, 1945; and DeBoer, Cadilek and Walters, 1955) and so on. However, the clinical trials with the γ-globulin prepared from convalescent human plasma to treat poliomyelitis in the preparalytic stage did not appear promising (Bahlke and Perkins, 1945). Consequently, wide application of specific antiserum in this disease was deemed impractical.

So far as is known, the function of antiserum in the treatment of a viral infection is only to prevent further spread of the pathogens. The cells which have already been invaded by virus probably will be completely destroyed even under adequate therapy. Of greatest importance to the host is the number of susceptible cells which are protected from invasion by the virus. It is logical to assume that if a considerable number of the cells were kept intact by the serum, and particularly if the vital respiratory and circulatory centers were protected, it would be possible for the host to live. Factors which are essential for achieving protection of as many cells as possible by antiserum are: early diagnosis of the particular disease, and prompt administration of the largest feasible dose of the serum intravenously. These principles have been practised in humans for the past half century on bacterial diseases, viz. diphtheria, tetanus.

As knowledge in the field of virology advances, it has been accepted that each virus behaves characteristically in its mode of spread, propagation in certain tissues, production of definite sequelae and the ultimate effect on its host. Therefore, the therapy for any given viral disease should be considered as an individual problem. According to Horstmann (1949) and Smadel and Adair (1952), poliomyelitis in man can be differentiated into 2 phases, the minor and the major. A case of poliomyelitis is diagnosed only after the beginning of the major phase, especially during an epidemic. In childhood-type poliomyelitis, the paralysis usually sets in 2–3 days after the onset of the major phase; whereas in the adult-type disease, paralysis may appear as late as 6–8 days after the major phase has started. Since Salk vaccine has become widely available, with special emphasis on the vaccination of children, it is reasonable to expect that cases of the adult-type would occur relatively more often than the childhood type. Hence diagnosis can be established early enough before the onset of paralysis to allow for administration of the serum.

Both the present and the previous (Liu *et al.*, 1958) reports deal with different aspects of serotherapy of poliomyelitis. This experimentally produced condition in which the CNS has been seeded with active virus is probably analogous to the commencement of the major phase of the disease in man (Bodian, 1955). The experiments previously reported (Liu *et al.*, 1958) were based on the assumption that any measure which can prevent the muscular paralysis 1–2 days before its occurrence would be a valid treatment for this disease in its pre-paralytic stage. In the current study, as mentioned in the beginning, emphasis was placed on the therapeutic angle. The experiments were designed to show whether or not the serum can modify the progression of muscular paralysis after it has begun. The success of serotherapy for such a condition depends upon not only the general principles just stated, but also the amount of specific antibody which can cross the blood-brain barrier. As discussed previously (Liu *et al.*, 1958), there appears to be a definite threshold existing for the antibody between the general circulation and the brain-tissue proper. This concept is confirmed particularly by the experi-

ments carried out in the current study. In the experiments using intracerebral challenge, 15 ml./kg. of the hyperimmune rabbit serum were solidly curative whereas 10 ml./kg. of the same serum were completely devoid of effect.

When an intracerebral challenge was used, the course of the experimental illness resembled the natural disease even more closely. The data presented leave no doubt that the serum treatment is effective in preventing further paralysis at any stage of the disease. When an adequate quantity of serum was given, the non-infected neurons in the CNS were protected. As shown in Figs. 1 and 2, the ascending paralysis was arrested completely within a period of 1–3 days according to the individual animals. The reasons for the delayed effect of the treatment may be twofold: a certain length of time is required for the antibody to reach the site of infection. As illustrated by the experiment summarized in Table I the neutralizing antibodies were dissipated evenly into the general circulation within a few minutes after intravenous injection. This has been confirmed by using ^{131}I labelled γ-globulin. However, 24–48 hr. were needed for a significant amount of the iodinated globulin to enter the brain tissue. These results will be reported in detail elsewhere (Liu and Carter, unpublished). And the neurons which had already been invaded by the virus ultimately degenerated.

It has been pointed out in the previous report (Liu *et al.*, 1958), that 3 factors may be responsible for the negative results of the therapeutic trials in poliomyelitic patients (Bahlke and Perkins, 1945). These factors are: low potency of the antiserum used, incorrect route of injection of the serum and existing high viral content in the CNS at the time of treatment.

Colio, Criley and Coriell (1958) reported recently that they prepared a hyperimmune antipoliomyelitis γ-globulin with very high neutralizing titers against 3 types of Poliovirus hominis. However, when this was used for treating monkeys infected with Mahoney virus after the onset of paralysis, it failed to produce a clear-cut therapeutic effect. Their methods differed in several important respects from those used in this laboratory. The paralysis was induced by the intramuscular administration of undiluted poliovirus of high titre, so the 2 challenge methods are not comparable. More important, perhaps, are the 2 methods of administration of antibody, *i.e.*, intravenous against intramuscular. The intravenous route has 3 advantages over the intramuscular route: larger volumes can be given, higher antibody levels can be attained in the systemic circulation and therefore in the CNS and the time required to attain maximum levels in the CNS is much shorter since the intramuscularly administered antibody will require many hours to produce maximum serum levels in contrast to 10–15 min. for intravenously administered antibody. Other aspects of the distribution of antibody by various routes have been discussed by Stevens (unpublished). He concluded that only the intravenous and intracisternal routes held promise for the therapeutic use of antibody.

As indicated in the experiments in which intracerebral inoculation was employed, the infectious dose for each animal was approximately 50,000 times that used for the intraspinal infection in the majority of the experiments. As early as 3 days post-infection, abundant pathogens were found at all levels in the spinal cord of the monkey. In spite of the high content of virus present in the CNS, the disease was clearly milder than that produced by the intraspinal inoculation. This is shown by a longer incubation period, a prolonged course of illness, fewer paralyzed muscles, and lower mortality in the infected hosts. More-

over, in the disease thus produced, the serum treatment appeared much more efficacious. Therefore, it seems unlikely that the previous failure of sero-therapy resulted partially from the third factor, *i.e.* high content of virus in the CNS at the time of treatment. On the contrary, at least experimentally, the location in the CNS where the virus was first seeded plays an important role in the efficacy of therapy. There is as yet no satisfactory explanation for this phenomenon.

On the basis of the data presented in Fig. 4, the antibody derived from human sources appeared definitely effective in the treatment of monkeys infected intraspinally with poliovirus. However, difficulties may be anticipated if a large dose of γ-globulin is given by the intravenous route to man. For the purposes at the present time, e.g., study on the treatment of poliomyelitis in cynomologus monkeys infected orally or clinical trial in human cases, the hyperimmune human plasma (or serum) would be sufficiently adequate. The potency of these preparations can be improved by more intensive schedules of immunization with Salk vaccine for donors before bleedings and by careful selection of blood samples afterwards.

SUMMARY

A study was carried out on the effect of delayed treatment of poliomyelitis in rhesus monkeys. In two experiments, a total of 24 monkeys was infected intraspinally with 10–16 $TCID_{50}$ of Mahoney virus. At 48 hr. after infection, when all animals demonstrated various degrees of paralysis and half the number showed elevated rectal temperatures, 15 were treated intravenously with 20 ml./kg. of hyperimmune rabbit serum. At the end of 4 weeks, the mean mortality of the treated groups was 7 per cent as compared with 78 per cent of the 9 controls. Also, the degree of muscular paralysis in the treated animals was milder when compared with that of the controls.

Within a period of 3–7 days after intracerebral inoculation of the Mahoney virus (500,000 $TCID_{50}$ per animal), 10 monkeys were treated with varying amounts of the hyperimmune rabbit serum intravenously. The 6 animals which received 15 ml./kg. of the serum, even as late as 7 days post-infection, recovered completely, whereas the 4 monkeys treated with 5–10 ml./kg. of serum as well as the 5 controls developed extensive muscular paralysis.

Hyperimmune human γ-globulin was prepared from a pool of plasma obtained by bleeding 39 donors after 2 injections of Salk vaccine. Its efficacy was tested in monkeys infected with the Mahoney virus intraspinally. Its therapeutic effect was similar to that earlier observed with rabbit serum, suggesting that the quantity of antibodies, not the source, was essential in arrest of the polio infection in the CNS.

The authors wish to express their deep appreciation to Drs. Joseph Stokes, Jr., Werner Henle and Kingsley M. Stevens for their valuable suggestions throughout this work.

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EXPERIMENTAL PYELONEPHRITIS: THE EFFECT OF RENAL VEIN CONSTRICTION ON BACTERIAL LOCALISATION AND MULTIPLICATION IN THE RAT KIDNEY

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THERE has been no satisfactory explanation for the ability of *Bacterium coli* to proliferate in a kidney whose ureter has been occluded. It has been suggested that ureteric obstruction causes a slowing of the renal circulation and that this is fundamental for the localisation and subsequent multiplication of organisms.

The present experiment was designed to test the effect of renal vein compression on localisation and multiplication of *Bact. coli* in the rat kidney.

METHODS

The organism was the same as that used in a previous experiment on the effects of permanent and temporary ureteric obstruction (Brumfitt and Heptinstall, 1958) and the bacteriological procedures are fully described in that paper. Female albino Wistar rats (Glaxo) of approximately 200 g. were divided into 2 groups. In the first of these a silver ribbon clip of 0·3 mm. calibre, such as was used by Wilson and Byrom (1941) for producing hypertension, was applied to the left renal vein through a flank incision under ether anaesthesia. After 30 min. 0·5 ml. of the bacterial suspension (approximately 200×10^6 organisms) was injected into the jugular vein under light ether anaesthesia. At fixed time intervals after injection, heart blood was taken and right and left kidneys removed for bacterial counting. The second group of rats consisted of paired control animals in which exactly the same procedure was followed as in the first group except that the left renal vein was merely separated from the artery but not clipped. The kidney was handled for the same length of time as in the first group.

RESULTS

The bacterial content of the blood was high at 15 minutes in both groups, falling rapidly with virtual sterility at 6 hr. Thereafter the blood was invariably sterile in all animals.

The Table shows the bacterial counts of right and left kidneys in both groups. With occasional exceptions the counts at 15 and 60 min. were similar in the left kidney with the vein clipped, the opposite kidney of the same animal, and in the non-clipped left kidneys of the control group. At 6 hr., 3 out of 6 clipped kidneys had considerably higher counts than either the opposite kidney of the same rat or the non-clipped left kidneys of the control group. At 2 days, 3 out of 7 and at 7 days, 6 out of 8 of the clipped left kidneys showed high bacterial counts compared with the opposite kidney of the same rat and with left kidneys in the control series. A solitary left kidney at 7 days in the control group showed a high count.

Certain rats had thrombosis of the left renal vein with death of kidney substance. These were excluded from the experiment, but it should be stated that all showed very high counts.

DISCUSSION

The concept that slowing of the renal circulation is responsible for increased trapping of organisms in the kidney, has been used to explain the ease with which

TABLE.—*The* Bact. coli *Content of Right and Left Kidneys in Rats with Silver Clip on Left Renal Vein and in Rats with no Clip*

Time of killing	Rats with clip on left renal vein		Rats with no clip on left renal vein (control group)	
	Right kidney	Left kidney	Right kidney	Left kidney
15 min.	20,000	23,000	20,000	21,000
	17,200	49,200	20,000	29,000
	29,600	26,400	24,800	24,000
	29,000	40,600	30,000	28,000
	57,200	226,000	56,800	31,400
	37,800	54,800	28,600	13,600
60 min.	8,700	25,000	3,800	5,800
	9,200	7,400	9,800	7,400
	18,200	53,600	13,200	13,400
	9,940	15,200	6,240	39,200
	7,280	11,200	9,100	11,200
	8,360	15,800	4,140	4,600
6 hr.	2,800	52,000	8,200	6,400
	3,240	8,600	3,840	22,200
	3,320	754,000	3,500	6,800
	3,060	4,620,000	2,600	5,000
	14,600	12,600	3,600	3,400
	2,600	9,800	4,160	4,600
2 days	6,060	9,200,000	1,380	800
	7,200	3,600	6,400	3,200
	280	0	6,700	16,000
	5,740	600	2,200	24,000
	24,200	9,080,000	0	0
	200	740,000	4,000	1,200
	300	1,000	80	400
7 days	300	60,000,000	800	1,000
	4,260	14,400	1,200	1,000
	6,200	108,000	0	0
	49,200	9,580,000	20	68,200
	0	43,400	1,300	0
	4,420	0	0	0
	20	540,000,000	800	800
	0	3,780,000	0	0

Bact. coli when injected intravenously localises in the kidney whose ureter is obstructed (Asher and Sokol, 1941). Similarly Navasquez (1956) has invoked slowing of the circulation, with increased trapping of organisms, to explain the development of *Bact. coli* infection in rabbit kidneys scarred by a previous injection of coagulase positive staphylococci. In a previous experiment (Brumfitt and Heptinstall, 1958) we confirmed the findings of Guze and Beeson (1956)

that increased trapping of organisms does not occur in the kidney of the rat with complete ureteric occlusion when *Bact. coli* are injected intravenously. It was found that in such kidneys, proliferation of organisms did not begin till between 2 and 6 hr. after their injection, and because of this we pointed out that even if ureteric obstruction does cause an appreciable slowing of the circulation (Lucas, 1908), then it has little effect on the initial deposition of organisms. We were in no position to answer the question of whether such a circulatory slowing might be responsible for the later proliferation of organisms. The present experiment demonstrates quite clearly that in the early stages (15 and 60 min.) the bacterial content of the clipped left kidney is not grossly different from its opposite kidney nor from the left kidneys in the control group. This latter group was included to eliminate the possibility that the mere handling of the kidney might cause an increased deposition of organisms, for Braude, Shapiro and Siemienski (1955) have shown that renal massage for 5 min. in the rabbit will produce pyelonephritis after the injection of *Bact. coli*. That the minimal handling of the kidney necessary to separate the renal vein from artery prior to applying the clip has no appreciable effect on the development of renal infection, is shown by a comparison of the left kidney in the control group with the right untouched kidney.

At both 6 hours and 2 days increased bacterial counts are present in half the clipped kidneys, and in 6 out of 8 at 7 days. In general the increase in bacterial count in kidneys whose renal vein has been constricted is neither so great nor so consistent as in kidneys whose ureter is obstructed. This lack of consistency is no doubt due to the difficulty in producing adequate renal vein obstruction without at the same time causing renal death. A clip of 0·3 mm. calibre was used because in a preliminary experiment this was found to be the smallest size that could be used without producing a high proportion of infarcted kidneys. Even when a clip of this size was used, thrombosis of the renal vein and necrosis of kidney tissue were found in several rats which were omitted from the experiment. However, it is probable that in some of the clipped kidneys the degree of constriction was not sufficiently great to alter the circulation. In no kidney included in the final evaluation was there naked eye evidence of infarction.

It can thus be stated that although constriction of the renal vein causes increased bacterial counts in the treated kidney following intravenous injection of *Bact. coli*, this increase is not due to an initial increased trapping of organisms, nor is vein constriction so potent a factor as ureteric obstruction.

SUMMARY

The left renal vein of rats was constricted by a clip, followed by injection of *Bact. coli* into the jugular vein. A control group in which the left renal vein was mobilised but not clipped was also injected with *Bact. coli*. In the early stages (15 and 60 min.) there was no great increase in the bacterial count of the kidneys whose vein had been constricted, but by 7 days three-quarters of the kidneys so treated showed increased bacterial counts. No similar increase was noted in the left kidney of the control group.

We are extremely grateful to Miss Jennifer Gathercole and Mr. Michael Jacobs for their willing technical assistance.

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OBSERVATIONS ON THE INTERACTION OF POLIOVIRUS AND HOST CELLS *IN VITRO*: II. THE ACTION OF INSULIN

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We have previously reported that an incubation temperature of 39·5° increases the production of poliovirus in tissue cultures (Likar and Wilson, 1958); the present paper describes experiments which show that the addition of insulin to tissue cultures has a similar effect. This was demonstrated by growing various primate tissues in media containing insulin, infecting these cultures with poliovirus and titrating the virus released at various intervals after infection.

MATERIALS AND METHODS

The methods described in a previous communication (Likar and Wilson, 1958) were used for the majority of the experiments presented here. Human amnion cells grown in medium 150 (Morgan, Campbell and Morton, 1955), HeLa cells grown in lactalbumin hydrolysate medium (Melnick, 1955) and rhesus monkey kidney grown in Maitland type cultures were used in addition to monolayers of second-passage rhesus monkey kidney cells.

The various types of tissue cultures were grown for 1 to 4 days in media containing concentrations of insulin between 0·001 to 5 i.u. per ml. At daily intervals this medium was removed and replaced with medium containing freshly added insulin in the appropriate concentration. After growth in this medium the tissue cultures were infected with type 1 (Mahoney or Brunhilde) poliovirus to give a final virus concentration in the medium of 10^3 to 10^4 TCD_{50}. There was no further change of medium after the inoculation of virus.

At various intervals after inoculation the infected tissue culture fluids from 6 to 8 tubes were harvested and pooled. All pooled fluids in any one experiment were stored at − 20° for periods of 2 to 8 weeks and were then titrated in tissue cultures of the same cell type as that in which the virus had been grown. All pools from any one experiment were titrated at the same time. Controls were set up with each experiment, in which the procedure was exactly the same except that no insulin was added to the tissue culture fluids.

The insulin which was used was crystalline insulin with approximately 23 i.u. per mg. and was kindly supplied by Boots Pure Drug Co. and Burroughs Wellcome & Co. It was dissolved in distilled water at pH 3 and was kept at 4° as a stock solution containing 45 i.u. per ml. A solution of Boots' insulin without preservative containing 40 i.u. per ml. was obtained through the courtesy of Dr. I. Leslie, Biochemistry Department, The Queen's University of Belfast.

A number of variables were tested to define the optimal conditions for the action of insulin on the poliovirus-host cell interaction. It was found that in monolayers of second-passage rhesus monkey cells grown in stationary tubes the following conditions influenced the results obtained; the duration of treatment of the tissue cultures with insulin prior to inoculation with virus; the concentration of insulin used for the treatment of the cells; the incubation temperature of the cultures. The effect of insulin on virus production by HeLa cells, human amnion and Maitland cultures of monkey kidney was also tested. The Brunhilde strain of type I poliovirus was used for the experiments in HeLa cells, all other experiments were made with Mahoney type I virus.

* Research Fellow, National Fund for Poliomyelitis Research, on study leave from the University of Ljubljana, Yugoslavia.

RESULTS

Time of treatment of tissue cultures with insulin prior to infection

Tissue cultures were grown for 18, 42, 66 and 80 hr. in a medium containing one i.u. insulin per ml. and were then infected with 10^3 TCD_{50} of poliovirus. There was no difference in the amount of virus produced by cells treated for 18 hr. with insulin as compared with controls.

In tubes treated for 42 hr. with insulin (Fig. 1) higher titres of virus were obtained than in control tubes, but the difference in the maximum titres reached was not significant. On the other hand, treatment of cells with insulin for 66 or for 80 hr. (Fig. 2) prior to infection, significantly increased the maximum titres of virus production. The maximum titre obtained in tubes treated with insulin for 66 and 80 hr. was 8·2 logs as compared with 7·2 in controls ($0{\cdot}02>P>0{\cdot}01$).

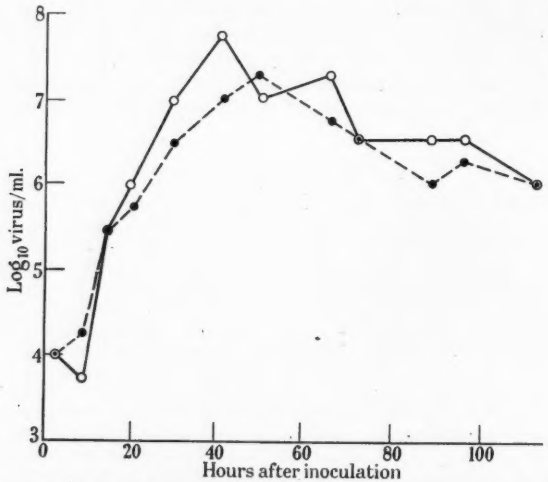

FIG. 1.—○———○ Titres of poliovirus in fluids of tissue cultures treated for 42 hr. with insulin.
●- - -● Titres of poliovirus in controls.
Each point on the graph represents the titration of the pooled fluids from 6–8 tubes.

Concentration of insulin

Cultures were grown at 37° in media containing 0·001, 0·01, 1 or 5 i.u. insulin per ml. After 80 hr. growth they were infected with virus and the fluids were then harvested at similar intervals to those in the experiment described above. The virus content of 14 pools at each insulin concentration was compared with controls to which no insulin had been added. The maximum titres obtained were as follows :

Insulin concentration/ml. medium	Maximum titres
5	8·2
1	8·2
0·01	8·0
0·001	7·0
Control	7·2

It will be seen that the maximum titres in fluids from cultures treated with 0·01, 1 and 5 i.u. of insulin were higher than in the control. The probability that these differences were due to chance was $< 0{\cdot}02$ in cultures treated with 5 i.u. or 1 i.u. of insulin and $< 0{\cdot}05$ in the cultures treated with 0·01 units.

Environmental temperature

In this experiment tissue cultures were grown in medium containing 1 i.u. insulin per ml. and incubated at 30, 37, 39·5, 40·5° or at room temperature for 66 hr. before inoculation with 10^3 TCD_{50} poliovirus.

The results of this experiment in which a total of 67 pools of tissue culture fluids were titrated are presented in Table I.

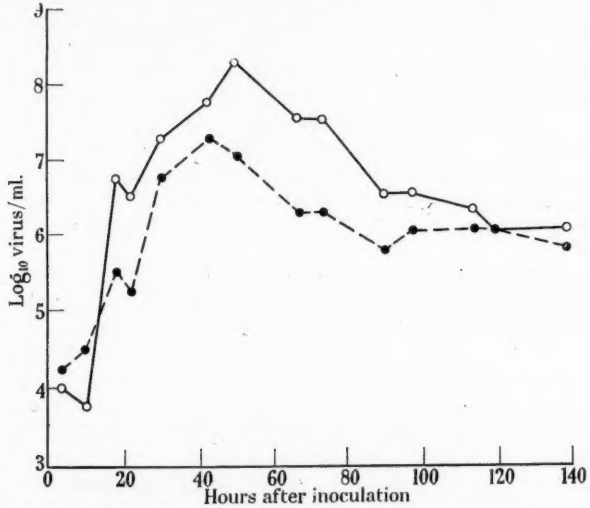

FIG. 2.—○——○ Titres of poliovirus in fluids of tissue cultures treated for 80 hr. with insulin.
●- - -● Titres of poliovirus in controls.
Each point on the graph represents the titration of the pooled fluids from 6–8 tubes.

TABLE I.—*Influence of Environmental Temperature on Virus Production by Tissue Culture Cells Grown in Medium Containing One Unit of Insulin per ml.*

Incubation temp. °	Maximum titres in tissue cultures: Without insulin	With insulin
Room (18–22) . .	4·5	5·0
30 . .	6·0	7·0
37 . .	7·2	8·2
39·5 . .	8·0	8·0
40·5 . .	5·0	5·0

It will be seen as already described (Likar and Wilson, 1958) that increase of temperature by itself (between room temperature and 39·5°) increases the production of virus. However the effect of insulin at various temperatures (Table I)

was operative only below 39·5° at which temperature there was no increase in the maximum virus titres reached in the insulin treated cultures compared with the controls.

Type of tissue used

A comparison was made of the effect of insulin on the production of virus grown in monolayers of monkey kidney, HeLa cells and human amnion and in monkey kidney Maitland cultures. All these cultures were grown at 37° for 80 hr. in media containing 1 i.u. of insulin per ml. before being infected with poliovirus. Control cultures were set up in which no insulin was used in the medium. The results of this experiment are shown in Fig. 3.

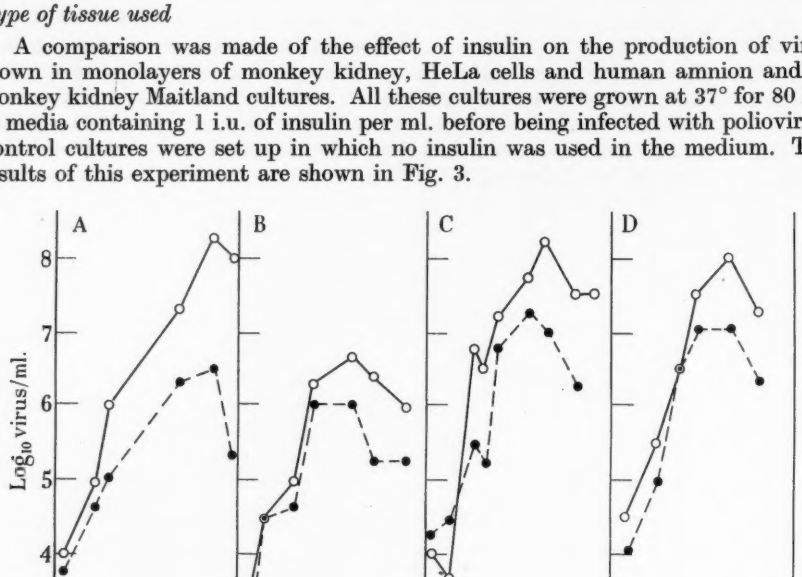

FIG. 3.—○———○ Titres of poliovirus in fluids of tissue cultures treated with insulin. ●- - -● Titres of poliovirus in controls.
A: Human amnion cells. B: HeLa cells. C: Monkey kidney monolayers. D: Maitland culture of monkey kidney.
Each point on the graph represents the titration of the pooled fluids from 6–8 tubes.

In all cases higher maximum titres were obtained when tissue cultures were treated with insulin. The differences in the maximum titres with and without insulin are shown in Table II.

TABLE II.—*Maximum Titres Obtained in Various Tissue Cultures Treated with Insulin and in Control Cultures*

Type of tissue culture	Maximum titre in tissue cultures	
	Without insulin	With insulin
Monkey kidney monolayer .	7·2	8·2
Maitland culture . .	7·0	8·0
HeLa cells . . .	6·0	6·8
Human amnion . .	6·4	8·2

DISCUSSION

Under the conditions of the experiments described, it has been shown that the addition of insulin to tissue culture media results in an increased production of virus when tissue cultures are infected with type I poliovirus.

This effect may be due either to an increased volume of cells in the insulin treated cultures or to a biochemical effect or to both. We have not yet determined cell counts or cell volumes in the insulin treated cultures but the fact that the insulin effect was observed in Maitland cultures suggests that the increase in virus production in tissue cultures treated with insulin cannot be explained simply by an increase in the number of cells. Furthermore the insulin effect was abolished at incubation temperatures of 39·5° suggesting that the effect may be enzymatic.

It has been shown by Eagle and Habel (1956) and Becker, Grossowitz and Bernkopf (1958) that increased glucose uptake occurs during the synthesis of poliovirus. It is possible that the insulin effect observed in our experiments may be due to an increase in the rate of transfer of glucose into the cells of insulin treated cultures, which results in increased virus production.

Leslie, Fulton and Sinclair (1957) have shown that increased synthesis of protein, nucleic acids and phospholipids occurred in tissue cultures treated with insulin. Increases in those substances rather than in the rate of glucose transfer might explain the greater virus production in insulin treated cultures in our experiments.

We have shown that virus production by monkey kidney monolayers incubated at 39·5° has an effect of increasing the virus produced by cells similar to that of insulin added to cultures incubated at 37° but that this temperature effect is not further enhanced by the addition of insulin.

Although further research is required to elucidate the action of insulin in virus production by tissue cultures, it may be of some immediate practical use. It is considered that the use of insulin-containing media could be successfully applied to increase the production of virus in the manufacture of poliovirus vaccines. Alternatively, the use of higher incubation temperatures could be used to produce a similar effect.

SUMMARY

The addition of insulin to culture media increases the production of type I poliovirus grown in monolayers of monkey kidney, HeLa and amnion cells and in Maitland cultures of monkey kidney.

The maximum titre of virus was obtained in monkey kidney monolayers which had been grown for at least 66 hr. in a medium containing 0·01 to 5 i.u. of insulin per ml. of medium, before being inoculated with poliovirus. The enhancing effect of insulin on virus production was abolished when an incubation temperature of 39·5° was used. Incubation at this temperature, in the absence of insulin, also increased virus production as compared with incubation at lower temperatures.

It is suggested that, in the manufacture of poliovirus vaccines, the addition of insulin to the culture media or higher incubation temperatures might be used to increase virus production in the tissue cultures.

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THE HISTOLOGICAL LOCALIZATION OF RENIN WITH FLUORESCENT ANTIBODY

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THE precise site of production of the pressor enzyme renin is unknown. Since the suggestion by Munoz, Braun-Menendez, Fasciolo and Leloir (1939) that the renal pressor substance of Tigerstedt and Bergman (1898) is a specific proteinase, many attempts have been made to decide where it is produced in the kidney. It is absent from renal medulla but opinions differ widely about the probable component of cortex responsible for its formation.

Goormaghtigh's (1939) demonstration that the juxta-glomerular apparatus in ischaemic kidneys of rabbits and dogs shows microscopical changes suggesting secretory activity, led him to suppose that this structure produces a pressor substance. The hypothesis is supported by a finding of Marshall and Wakerlin (1949) that the amount of renin extractable from dog kidney is correlated with the granularity of the juxta-glomerular apparatus. On the other hand a relationship has also been observed between renin assay and tubular proliferation in developing foetal pig kidneys (Kaplan and Friedman, 1942). The further observation that renin could not be demonstrated in rabbit kidneys in which the proximal tubules had been selectively destroyed by the administration of sodium tartrate (Friedman and Kaplan, 1943) suggested to these authors that the proximal convoluted tubules are the site of renin production or storage. This fits with Taquini's (1950) finding from indirect assay of sections of kidney from the dog, cat, ox and pig, that renin was only demonstrable in the outer 1·5 mm. of cortex which consists of convoluted tubules. However, Yoshimura and Negishi (1954) in similar studies to those of Friedman and Kaplan, were unable to repeat their findings. Furthermore, Cook, Gordon and Peart (1956), by direct assay of sections of rabbit kidney, found renin only where there were glomeruli; there was none in the subcapsular zone. This work had been extended by Cook and Pickering (1958) who devised a magnetic filter to concentrate magnetised glomeruli obtained from kidneys perfused with a suspension of magnetic iron oxide; they found the highest concentration of renin in the fraction containing large glomerular fragments and attached renal tubular segments. Bing and Wiberg (1958) by microdissection and direct renin assay of cortical components of pig, rabbit and rat kidney, also concluded that the glomerular zone was richest in renin though none was detected in the capillary tufts themselves.

The known antigenic properties of renin (Johnson and Wakerlin, 1940) prompted us to try another line of investigation using the fluorescent antibody technique. A tissue antigen may be specifically identified microscopically by virtue of its affinity for the corresponding antibody labelled with a fluorescent

dye. The method, recently reviewed by Coons (1956) and Cruickshank and Currie (1958), has already been applied to the histological localization of normal tissue components, *e.g.* adrenocorticotrophic hormone in the pituitary (Marshall, 1951), kidney antigens (Hill and Cruickshank, 1953) and pancreatic enzymes (Marshall, 1954). The fact that pure renin has not yet been obtained presents difficulties in that a single specific antibody required ideally for the technique is unobtainable. Nevertheless, we have found that many, though not all, of the problems posed by the heterogeneous nature of the antigen and corresponding antiserum may be overcome by suitable serum absorptions. The sensitivity of the fluorescent antibody method is increased by the use of the " immunological sandwich method " in which specific antibody globulin applied to the corresponding tissue antigen is identified by means of a second layer of fluorescent serum antiglobulin. The extra sensitivity thus obtained has been further enhanced in the present experiments by applying the recently introduced technique with lissamine rhodamine B200 (RB 200) as the fluorescent tracer (Chadwick, McEntegart and Nairn, 1958*a* and 1958*b*). These refinements of method have enabled us to show that highly absorbed antirenin serum has an affinity for glomerular tissue which seems to be specific ; the histological findings suggest that the glomerular epithelium may be the site of renin production or accumulation in the renal cortex.

METHODS

The following steps were taken : (1) Preparation of purified renin extract from pig kidneys. (2) Production in rabbits of antiserum to the pig renin. (3) Removal of all precipitins from the antirenin serum by absorption with pig tissue powders, homogenates or renin extract. This left a proportion of the antirenin activity ; the renin/antirenin complex is non-precipitating (Lamfrom, Haas and Goldblatt, 1954) and remains in solution when the renin extract is used for precipitation. (4) Labelling of antiserum. Attempts at direct conjugation of the antiserum with either fluorescein (Coons and Kaplan, 1950) or with RB 200 resulted in a variable loss of antirenin activity, always more than 50 per cent and particularly serious here because the original titre was poor. The direct tracer technique was therefore abandoned in favour of the " sandwich method " with a potent anti-rabbit globulin prepared in a goat. Conjugates of this serum with RB 200 were used for the main study. Comparative experiments with fluorescein labelling gave similar results, but this tracer was found to be insufficiently sensitive for general use with the fully absorbed antirenin serum. (5) Fluorescence microscopy of sections of pig kidney and other organs treated successively with the rabbit antirenin serum and the RB 200-labelled goat antirabbit globulin.

Preparation of renin

The method of Haas, Lamfrom and Goldblatt (1953) was used. Sample (1) used as antigen for the preparation of the antirenin serum was a gift from Dr. Haas ; it had a potency of 90 Goldblatt units/mg. N_2. Sample (2) required for other purposes during the investigation, when the first was exhausted, had a potency of 33 units/mg. N_2.

Production of antirenin

Because pure renin extract was scarce, only two rabbits, adult female chinchills weighing 3 kg., were used to make the antibody; a usable antirenin titre was obtained in one animal after 2 immunizing courses with adjuvant. In the first course, three 27 unit doses of pig renin in Freund's adjuvant excluding *Mycobacterium tuberculosis*, were divided equally in subcutaneous and intramuscular sites and administered at 11 day intervals. Two months later, 5 further 27 unit doses of renin in saline solution were given intramuscularly at approximately 3 week intervals. Serum samples were tested before each inoculation for antirenin activity and for precipitins. The latter, unrelated to the non-precipitating antirenin and

attributable to renal components in the antigen other than renin, provided an extra index of the rabbit's immunological responsiveness to pig protein. The yield from this first immunizing course was poor; most serum samples showed 1 or 2 units of antirenin activity/ml. and only once did the titre rise to 4 units/ml.; one of the rabbits gave a consistently poorer response, about half that of the other rabbit.

After this first immunizing course, it happened that the rabbits were rested for a year and then they were given a second course beginning with 80 units renin in Freund's adjuvant including *Myco. tuberculosis*, intramuscularly, in multiple sites, followed a month later by 8 daily injections of 5 units renin in saline solution intraperitoneally. Five days after the final injection, the antirenin titre in the more responsive rabbit had risen to 20 units/ml., in the other to 10 units/ml.; the precipitin titre in both sera, tested with sample (2) renin at a concentration of 2 units/ml., was 1/20. Except for a few comparative studies, the serum with the higher antirenin titre has been used for all the experiments to be described. Antirenin assay was carried out by the method of Lamfrom, Haas and Goldblatt (1954) using, instead of dogs, "trained" rabbits kept quiet and warm while the blood pressure was recorded in the ear with a Grant capsule (Grant and Rothschild, 1934). The pressor response of the rabbits to renin of known strength, in Goldblatt dog units, was checked frequently and only animals giving consistent and reproducible results were used for bio-assay.

Immunological absorptions of antirenin serum

The precipitins in the antirenin serum tested against sample (2) renin in saline were readily identified by the ring test. They could be reduced by absorption of the serum with acetone-dried pig liver powder, serum powder, kidney medulla powder or wet packed homogenate of medulla; they were eliminated by absorption with kidney cortex powder or homogenate, or sample (2) renin at optimum precipitating proportions (11 units renin/ml. antirenin serum). Further investigation of the precipitins was carried out by simple diffusion of antigen (sample (2) renin, 2–10 units/ml.) into antiserum at 1/5 dilution in 1 per cent agar columns of 1·5 mm. bore (Oudin, 1952). Eight diffusion bands appeared in 48 hr. at room temperature; there was subsequent increase in complexity which could not be readily analysed. Four of the major bands, including the two with greatest density failed to appear when the serum had been absorbed with liver or kidney medulla powders; there were no diffusion bands after absorption of the serum with kidney cortex or renin at optimum precipitating proportions.

Exact determinations of the antirenin activity of absorbed sera were not always possible because of scarcity of serum, but the expected changes occurred. There was little or no reduction in antirenin titre after absorption with pig tissues other than kidney cortex which caused about a 65–80 per cent loss of activity; the acetone-dried cortex powder was more potent in this respect than the wet homogenate. Absorptions with renin at optimum proportions resulted in virtual arithmetical reduction in antirenin titre, *i.e.* removal of precipitins from 1 ml. of serum containing 20 units of antirenin with 0·28 ml. (11 units) of a solution of sample (2) renin, left 9 units (7 units/ml.) of antirenin activity in the supernatant.

In practice, most absorptions were carried out by shaking the sera with the pig tissue preparations (0·1 g./ml. for powders and 0·5 ml./ml. for homogenates) in a mechanical agitator for 1 hr. at room temperature, followed by centrifugation at 10,000 r.p.m. for 30 min. to recover the serum. Sometimes sera were absorbed more than once with the same or different tissue preparations; occasionally, in particular for the precipitation with renin extract, the reaction time was prolonged by incubation for 1 hr. at 37° and refrigeration overnight before centrifugation.

Fluorescent labelling of goat serum (anti-rabbit globulin)

The serum from a hyperimmune goat had an anti-rabbit globulin precipitin titre of 1/20 by the ring test against 1/1000 rabbit globulin; at a 1/5 concentration, it detected rabbit globulin at dilutions up to 1/10,000. Immediately after conjugation with RB 200, it was treated with powdered activated charcoal to remove dye not chemically attached to the serum proteins and then successively with the following acetone-dried pig tissue powders to remove non-specific antibodies: liver, kidney cortex and alcohol-denatured serum. The absorbed conjugated serum globulin was precipitated with ammonium sulphate (40 per cent saturation), the precipitate dissolved in a volume of saline equal to the original serum

volume, and the solution dialysed overnight against buffered saline at pH 7. This conjugate gave brilliant orange fluorescent staining with test bacteria coated with rabbit antisera in smears and sections but had no staining affinity for kidney tissue.

Preparation and microscopy of sections

Kidneys were taken from healthy adult male and female pigs within 20 min. of slaughter; small blocks (5–10 mm. across and 2 mm. thick) of kidney were quick-frozen in containers immersed in alcohol dry-ice freezing mixture and stored at −20°. Frozen sections were cut at 5–6 μ in a low temperature freezing cabinet at −18° to −20°, and mounted on formol-gelatin coated slides (Coons and Kaplan). After drying for 1 hr. in a current of air, the sections were fixed in 95 per cent ethanol at room temperature. Fixation of the antigen is particularly important in the present experiments because of the need to locate an antigen-antibody complex which is soluble under normal conditions. Unfixed sections gave unsatisfactory ill-defined fluorescence with the unabsorbed antiserum and no significant fluorescence after the precipitins had been removed. To decide the best method of fixation, other fixatives including methanol, absolute ethanol, isopropanol and several other higher alcohols, acetone, ether and weak solutions of formalin were tried alone or in combination and at various temperatures. Of these only isopropanol gave satisfactory fixation without denaturation of test renin smears, permitting identification with fluorescent antibody; but somewhat better results were obtained with 95 per cent ethanol and, though not an ideal protein fixative, this was adopted as a routine. After fixation, sections were again dried in a current of air at room temperature for 30 min. and were ready for staining. This was usually performed the same day, occasionally on the following day after storage of the sections at 0°; longer storage sometimes gave anomalous results and old sections were therefore discarded. Sections were treated with a drop of antirenin serum for 60 min. in a damp container to reduce evaporation; after rinsing and washing for 10 min. with buffered saline (pH 7) they were treated with RB 200-conjugated goat anti-rabbit globulin for 20 min., washed again in buffer for 10 min. and mounted in buffered glycerol. Sections prepared in the same manner from other pig organs including liver, lung, heart, and adrenal, and from kidneys of mice were also examined from time to time.

Fluorescence microscopy of the sections was carried out as soon as possible after mounting, using the Reichert equipment modified for ultraviolet-blue light with a yellow filter above the object (Nairn, Chadwick and McEntegart, 1958; Chadwick *et al.*, 1958*b*). Specific orange fluorescent staining of tissue antibody contrasted well with the fainter greenish-khaki background due to tissue autofluorescence. Absence of staining in tissues treated with (*a*) normal rabbit serum or (*b*) antirenin serum fully neutralized with renin antigen, provided tests of specificity.

RESULTS

These are summarized in the Table in which the staining of tissue sections treated with antirenin serum followed by fluorescent goat anti-rabbit globulin is a measure of the affinity of the serum for the tissues. The unabsorbed anti-

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EXPLANATION OF PLATE

Fluorescence photomicrography prints from "Super Anscochrome" colour transparencies.

FIG. 1.—Frozen section of pig kidney, fixed in 95 per cent ethanol and treated first with antirenin serum absorbed with pig liver powder, and then with RB 200-conjugated goat anti-rabbit globulin. Specific fluorescent staining is conspicuous in the cells of the glomerular tuft and capsule; it is also just distinguishable in the peritubular ground substance and blood vessels. The fluorescence in the tubules is solely autofluorescence. × 120.

FIG. 2.—High-power view of similar preparation to Fig. 3 in which the antirenin serum had been absorbed with pig liver and kidney cortex powders. It shows a single glomerulus with less bright fluorescent staining than in Fig. 1, but the same cellular distribution can be made out. The specific cytoplasmic staining can be seen around non-fluorescent nuclei, and the fluorescence of the surface epithelium can be made out near the lower left corner. The bright yellow spots are fluorescent artefacts used as an aid to focusing. ×280.

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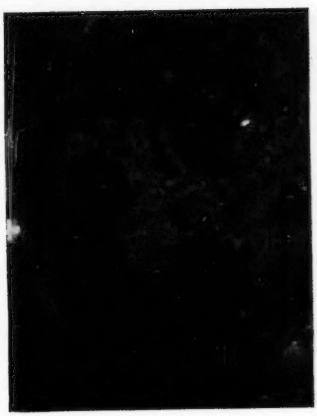

2

Nairn, Fraser and Chadwick.

TABLE.—*Reactions of Unabsorbed and Absorbed Antirenin Serum and of Normal Serum*

Absorptions with pig tissue preparations	Antirenin rabbit serum: *Nil*	Liver powder	Kidney medulla powder	Liver powder + Kidney medulla powder	Liver powder + Kidney cortex powder	Liver powder + Kidney cortex homogenate	Renin: Optimum precipitating proportions	Renin: Neutralizing antirenin activity	Normal rabbit serum Liver powder + kidney cortex homogenate
Specific staining with labelled goat antirabbit globulin—									
Kidney glomeruli	+++	+++	++	++	±	+*	±	−	−
Tubules etc.	++	+	±	±	−	−	−	−	−
Other organs	+	±	±	v	v	v	v	v	(v)
Antirenin activity† (units/ml.)	18–20	15–20	..	12–20	3–5	5–8	5–8	0	0
Gel diffusion rings at 48 hr. against renin	8	4	4	..	0	0	0	..	0

* Staining of glomeruli unchanged by a second absorption with homogenate but blocked by neutralizing antirenin activity with renin (8 units renin/ml. serum).

† Only ranges available because of scarcity of material for bioassay.

v Slight and inconstant staining mainly lung and adrenal medulla.

renin serum had strong affinity for kidney tissue, especially the glomeruli, and also some affinity for control tissues (liver, lung, heart and adrenal), but the staining was diffuse and lacked definition. This lack of organ and tissue specificity is not unexpected because the antiserum clearly contains at least 8 precipitating components besides the non-precipitating antirenin. Removal of 4 of the precipitins by a single absorption with pig liver powder caused little or no diminution of antirenin activity but reduced tissue staining in the kidney and in the control organs. In the latter, staining was faint but now more sharply defined and mainly limited to blood vessels and plasma. In the kidney, staining of glomeruli remained conspicuous but elsewhere was diminished and mainly confined to the peritubular ground substance and blood vessels. The appearances in the kidney are illustrated in Figs. 1 and 2 : the specific glomerular fluorescence has the distribution of the cellular elements not the basement membrane. It is not possible to distinguish epithelial and endothelial cells in the centre of the glomerular tuft in these preparations, but the peripheral fluorescence is almost certainly located in the tuft epithelial cells and there are a few fluorescent cells in the position of the capsular epithelium. Thus whilst the fluorescence may be entirely epithelial, the possibility of mixed epithelial and endothelial distribution cannot be excluded. There was no suggestion of specific staining of the " juxtaglomerular " area.

The results after a single absorption with kidney medulla powder were similar except that the kidney staining showed a more uniform reduction, though glomerular fluorescence was still conspicuous. After successive absorptions with liver powder and kidney medulla powder much the same picture was seen in the kidney whilst the staining in the control organs was now only trivial, mainly confined to blood plasma in the adrenal medulla and lung. The minor staining in these latter organs was a variable finding after all double absorptions and could

be neglected ; it was reduced almost to extinction, without effect on the kidney staining, by a further absorption with denatured serum powder, and was presumably partly non-specific since it appeared occasionally in control preparations treated with non-immune rabbit sera. Such normal sera when fully absorbed with pig liver and kidney cortex powder did not induce glomerular fluorescence or any significant staining elsewhere in the kidneys or other organs ; unabsorbed normal sera were avoided for control experiments because they were associated with variable minor non-specific staining of all organs.

Absorptions of the antiserum with liver powder followed by kidney cortex powder or homogenate removed all precipitins and reduced the antirenin activity

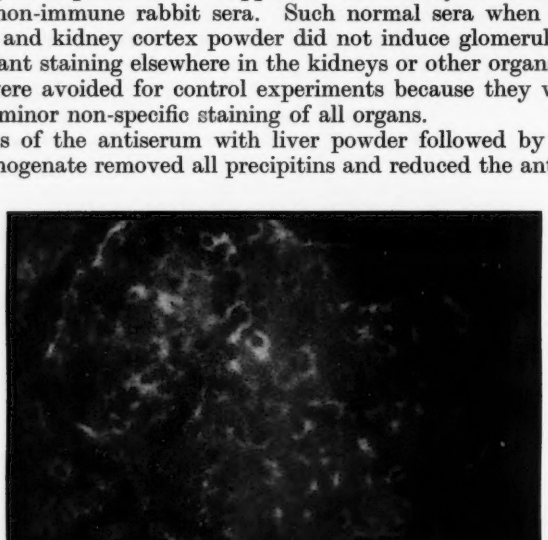

FIG. 3.—Black and white print from a "Super Ansochrome" colour transparency showing a single glomerulus in a similar preparation to Fig. 1. Cytoplasmic fluorescent staining can be seen around non-fluorescent nuclei and the fluorescence of the surface epithelium of the tuft is well shown. ×360.

to 3–8 units/ml. Tissue staining was also eliminated except in the kidney glomeruli where it was diminished but still recognisable (Fig. 3) ; there was no staining in the " juxta-glomerular " areas or in the tubules. The distribution of the glomerular fluorescence looked much the same as that induced by the partly absorbed antisera ; fluorescent cells corresponding in position to the tuft and capsular epithelium were again identified. The glomerular staining was not reduced by a second absorption with cortex homogenate, but when this thrice absorbed serum was treated with renin (8 units/ml.) to neutralize the remaining antirenin activity, its affinity for glomerular tissue was abolished. Similar results were obtained when the precipitins were removed from the serum by treatment with sample (2) renin at optimum precipitating proportions : the supernatant had an antirenin activity of 5–8 units/ml. and showed moderate and consistent affinity for glomerular tissue only. This affinity was blocked by neutralizing the antirenin activity with sample (2) renin.

Unsuccessful attempts were made to restore the glomerular affinity of renin-neutralized sera by recovery of antirenin with alkali : the serum was brought to

pH 11·3 for 1 hr. to detach and destroy the renin in the renin/antirenin complex, and then restored to pH 7 (Lamfrom *et al.*, 1954); in our hands, this resulted in only about 30 per cent recovery of antirenin activity. Absence of glomerular staining with such a reconstituted serum may perhaps be attributable to its final low antirenin titre combined with a possible blocking effect by the denatured renin which is still in the serum.

In brief, elimination of precipitins from the antiserum was associated with loss of general pig protein antibodies and of more specific kidney antibodies, but left a proportion of antirenin activity. This absorbed serum, when applied to kidney sections and followed by fluorescent anti-rabbit globulin, had the property of inducing faint but characteristic fluorescence limited to the cells of the glomeruli; the staining could be blocked by neutralising the antirenin activity of the serum with the calculated quantity of renin.

Mouse kidney sections treated with unabsorbed antirenin serum showed some generalized ill-defined staining which was decreased by absorption with pig liver and kidney cortex powders and abolished by further absorption with homologous tissue powder; the characteristic glomerular staining of the pig kidney was not seen. An attempt was made to introduce pig renin into mouse kidneys by the intravenous injection of 10 units of sample (2) renin and killing the mice at intervals up to 30 min. afterwards. Sections of these kidneys treated first with antirenin serum absorbed with pig liver powder and then with labelled goat anti-rabbit globulin, showed some specific fluorescent staining of the plasma; the staining was very faint but still detectable after re-absorbing the antirenin serum with pig kidney cortex powder. There was no sign of selective localization of the fluorescence in the renal tubules or glomeruli. This experiment is a further indication that the method is capable of locating the renin antigen at low concentration in the tissues.

DISCUSSION

The antibody in the antirenin serum, prepared against a purified sample of renin, has been shown to be predominantly anti-glomerular, as measured by the fluorescent tracer " sandwich " technique. The glomerular affinity of the serum, though reduced in parallel with the antirenin activity by immunological absorptions, was only abolished when this activity was completely neutralized. This is to say, the glomerular affinity and the antirenin activity of the serum could not be dissociated in these experiments. There are two possible explanations for this finding: either the glomerular affinity and the antirenin activity are due to the same antibody, or the serum contains, besides the antirenin, a non-precipitating anti-glomerular component with the same titre as the antirenin. The latter explanation is an unlikely one but cannot be excluded with certainty until either pure renin or pure glomerular proteins become available to carry out specific neutralizing blocking tests.

If it be accepted that the glomerular affinity and antirenin activity of the serum are likely to be due to the same antibody, then the specific glomerular fluorescence obtained with the absorbed serum may be attributed (*a*) wholly to renin, or (*b*) to glomerular protein molecules of which a proportion have renin activity, or (*c*) to glomerular protein, not renin, but indistinguishable immunologically from it. The first two of these hypotheses seem to be the more reason-

able : that the fluorescence obtained in the glomerular cells is an indication of the presence of renin.

The attempt to demonstrate specific fluorescence in mouse kidneys seemed worth making because information about species specificity of renin/antirenin reactions is incomplete and the possibility of a reaction between mouse renin and the antirenin prepared in the rabbit against pig renin, could not be predicted. In the event, the failure to obtain glomerular fluorescence, though an interesting finding, does not affect the conclusion already drawn from the findings in the pig kidney. On the other hand, the demonstration of faint specific fluorescence in the plasma of the mice that had been injected with the renin antigen provides further support for the view that the specific fluorescent staining of the pig kidney could also be due to the presence of this antigen.

We have not been able to determine by the present techniques whether the glomerular fluorescence obtained in pig kidney sections occurs only in the epithelial cells or whether it is also present in the capillary endothelium. It would be unwise to discount a possible secretory function of the endothelial cells since they have been shown by electron microscopy (Rhodin, 1958) to have a small Golgi zone and occasional α-cytomembranes, features usually associated with secretory activity as in pancreatic cells and mucus-producing cells. It might be feasible when a more potent antirenin serum becomes available to decide whether the endothelial cells in fact participate in the specific glomerular staining, by counterstaining the basement membrane of the tuft ; this could be done with a fluorescein-conjugated antiserum to pig reticulin.

SUMMARY

An antirenin serum (20 units/ml.) was prepared by immunizing rabbits with a purified extract of pig renin (90 units/mg. N_2) in Freund's adjuvant. Frozen sections of pig kidney, fixed in 95 per cent ethanol, were treated with the antiserum which becomes attached to the corresponding antigens in the tissue ; the antiserum was itself traced with a potent goat anti-rabbit globulin labelled with lissamine rhodamine B 200, and the sections were examined by fluorescence microscopy. The good contrast of the tracer with the tissue autofluorescence and the extra sensitivity of the " sandwich method " permitted the detection of specific fluorescence in the kidney after repeated immunological absorptions of the antirenin serum to remove all precipitins.

The fluorescent staining with unabsorbed antiserum was mainly glomerular and entirely so after removal of the precipitins. The glomerular fluorescence appeared to be located in the epithelial cells but the possibility of endothelial staining in addition, could not be excluded. Serum absorptions with pig tissue, including kidney cortex preparations, or with renin extract at optimum precipitating proportions, left a proportion of antirenin activity ; this antibody is non-precipitating. Glomerular staining was blocked by neutralizing the antirenin activity with renin extract ; absence of significant staining with fully absorbed antiserum in sections of pig liver, lung, adrenal and heart, and absence of kidney staining with normal rabbit serum provided further tests of specificity.

The results, which show that the antibody in the antirenin serum was predominantly anti-glomerular, suggest that renin is located in the glomeruli, probably in the epithelial cells. Whilst the evidence for this seems strong, it

cannot be conclusive with the present technique until pure renin becomes available to carry out a specific neutralizing blocking test.

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HETEROLOGOUS INTERFERENCE OF EXPERIMENTAL SERUM ANAPHYLAXIS IN GUINEA-PIGS: A PRELIMINARY REPORT

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THE suppression of antibody formation due to the overwhelming immune response to heterologous antigen, a phenomenon described by various authors under the terms of " Konkurrenz der Antigene " (Michaelis, 1904), " crowding-out " (Glenny, Hopkins and Waddington, 1925) and " heterologous interference " (Chen, Chou, Huang, Wang, Ko and Huang, 1956), has been the subject of study of several recent publications (Barr and Llewellyn-Jones, 1953; Abramoff and Wolfe, 1956; Chen *et al.*, 1956 and Adler, 1957). Theoretically, investigation of this phenomenon helps in revealing the mechanism of antibody formation. On the practical side the phenomenon has been found to play a role in Rh countersensitization (Shanaphy, 1952) and combined vaccination (Chen *et al.*, 1956 and 1957), and possibly in passive serum therapy also (Uhr, Pappenheimer and Yoneda, 1957).

In an effort to explore the mechanism of heterologous interference with antibody formation, experimental serum anaphylaxis has been used as a tool of study in this laboratory. The interference of experimental serum anaphylaxis in guinea-pig has not been extensively studied since the earlier work of Lewis (1915).

MATERIALS AND METHODS

Guinea-pig.—In-bred guinea-pigs raised in this laboratory were used. Groups of 5–6 pigs weighing 180–230 g. were caged separately and fed grain mixture, greens, and tap water *ad libitum*. Both sexes were used because the susceptibility to serum shock is identical (Coulson and Stevens, 1949). However, care was taken to keep the same male-female ratio in each group in any experiment, and the animals in each cage were of one sex.

Antigens.—Diphtheria and tetanus toxoids were the products of ammonium sulphate purification with a purity of 1903 and 1234 Lf respectively per mg. of trichloroacetic acid-precipitable nitrogen. The *Salmonella typhi* suspension was a formalin killed preparation made from strain H 901 which was devoid of Vi antigen, and the *Haemophilus pertussis* vaccine was a Merthiolate killed suspension prepared from phase I strains. The bacterial count of the former was standardized against MacFarland's nephylometric tubes and that of the latter against a Pyrex glass standard (obtained from N.I.H., Bethesda, Maryland, U.S.A.) in a Klett Summerson photoelectric colorimeter. Sheep red blood corpuscles (rbc) were obtained by subjecting the defibrinated blood to a centrifugal force of 400 *g* eight times for 10 min. each in about 15 volumes of saline. Frozen bovine serum after thawing was filtered through layers of cotton and gauze before use.

The toxoids and bovine serum were kept at — 20°, bacterial vaccines at 2–4°, and the sheep rbc suspensions were freshly prepared. Toxoids were used at a concentration of 10 Lf/ml., typhoid vaccine 10^9 organisms/ml., *pertussis* vaccine 5×10^9 organisms/ml., and

sheep rbc 20 per cent. Bovine serum was used for sensitization at a concentration of 1 per cent.

Pretreatment, sensitization and challenge.—Guinea-pigs were given a preliminary immunization course of toxoids, bacterial vaccines or sheep rbc between days 1–20. The preliminary immunization consisted of 2, 3, or 4 injections which were given on days 1 and 20 or 1, 10 and 20 or 1, 7, 14 and 20 respectively. On the 21st day the animals were sensitized with bovine serum. The route of both immunization and sensitization was intraperitoneal and the dose was 1 ml.

On the 41st day animals showing satisfactory weight gain were challenged with varying doses of bovine serum. The skin over the tendon of peroneus muscle where the short saphenous vein runs obliquely above was cut for a length of about 0·5–1·0 cm. to expose the vein and the shocking material introduced intravenously. The symptoms of the animals were followed for 3 hr. and the LD_{50} was calculated according to the method of Reed and Muench (1938).

Isolation and Nitrogen determination of γ-globulin.—The isolation of serum γ-globulin was performed by a modification of the low-temperature-ethanol-precipitation method (Weimer *et al.*, 1955). Nitrogen determination of serum γ-globulin and total serum protein was performed by the micro-Kjeldahl method described by Kabat and Mayer (1948). In both γ-globulin isolation and nitrogen determination, representative samples from each experimental group were included in each run in order to equalize as much as possible any unforeseen experimental error.

RESULTS

Experiment series I.—In experiments 1–5 the influence of pretreatment with tetanus toxoid, pertussis vaccine and sheep rbc on the development of hypersensitivity to bovine serum was studied. The general plan was to challenge 2 groups of 5 animals first at 0·7 log (5-fold) dose interval. If the trial challenge happened to bracket the LD_{50} in all the experimental groups, then the rest of the animals would be similarly challenged. Otherwise a new challenge dose of equal log distance would be added. The results of this experimental series are recorded in Table I.

It will be seen from Table I that serum hypersensitivity was consistently suppressed in the animals which received 2 doses of tetanus toxoid pretreatment. Under the conditions of the experiment, the toxoid-treated animals were 4·0, 7·1, 8·2, 9·0 and more than 10·0-fold respectively more resistant to serum anaphylaxis than the control animals, in terms of LD_{50}. In contrast, the development of serum hypersensitivity was hardly interfered with in the pertussis vaccine-treated group, even though 4 injections of vaccine were given. The greatest suppression of serum hypersensitivity in this group was observed in experiment 2 where the vaccine-treated group was 2·7-fold more resistant than the control; a value smaller than the smallest figure of 4-fold of the tetanus toxoid group. Even this degree of weak interference was not reproducible, since in duplicate experiment 3 the vaccine-treated group became more than 1·3-fold (by extrapolation the LD_{50} of the vaccine-treated group is estimated to be 0·2 ml. of 30× serum, accordingly a tentative sensitivity ratio figure of + 4·1 is obtained) more sensitive than the control, indicating that the small suppressive figure of 2·7-fold in a single experiment was probably fortuitous.

Under our experimental conditions the sheep rbc appears to be intermediate between tetanus toxoid and pertussis vaccine in its ability to interfere with the serum hypersensitivity in the guinea-pig. No interference was observed when the number of preparatory rbc injections was restricted to 2, whereas slight but detectable interference appeared when 3 or more injections were given. In the

TABLE I.—*Serum Sensitivity Ratios of Guinea-pigs Pretreated with Tetanus Toxoid, Sheep Red Blood Corpuscle and Pertussis Vaccine*

Exp. No.	Challenge dose, LD_{50} and sensitivity ratio	Saline control	Tetanus toxoid	Sheep rbc	Pertussis vaccine
1	Undil. 0·5 ml.	*2**	*2* 3/9	*2*	*2*
	2× 0·2 ml.	7/9†	0/9	7/8	8/9
	10× 0·2 ml.	1/8		3/8	1/9
	LD_{50}	4×0·2 ml.	>Undil. 0·5 ml.	6·1×0·2 ml.	4·5×0·2 ml.
	Sens. ratio§		> −10·0	+1·5	+1·1
2	Undil. 0·5 ml.	*3*	*2* 5/6	*3* 5/5	*3*
	2× 0·2 ml.	6/6	(*1*)‡ 3/6	4/5	6/6
	10× 0·2 ml.	4/6	1/6	2/5	2/6
	50× 0·2 ml.	1/6			0/5
	LD_{50}	18×0·2 ml.	2×0·2 ml.	6×0·2 ml.	6·7×0·2 ml.
	Sens. ratio		−9·0	−3·0	−2·7
3	Undil. 0·5 ml.	*3*	*2* 9/10	*3*	*3*
	2× 0·2 ml.	9/10	(*1*) 1/9	4/7	10/10
	10× 0·2 ml.	4/9		1/7	7/10
	LD_{50}	7·4×0·2 ml.	Undil. 0·22 ml.	2·9×0·2 ml.	>10×0·2 ml.
	Sens. ratio		−8·2	−2·6	>+1·3
4	Undil. 0·5 ml.	*4*	*2* 7/8	*4*	*4*
	2× 0·2 ml.	8/9	(*2*) 4/9	5/8	5/8
	10× 0·2 ml.	3/9		1/9	2/9
	LD_{50}	6×0·2 ml.	1·5×0·2 ml.	3·2×0·2 ml.	3·7×0·2 ml.
	Sens. ratio		−4·0	−1·9	−1·6
5	Undil. 0·5 ml.	*4*	*2* 6/9	*4* 6/9	*4*
	2× 0·2 ml.	7/9	(*2*) 3/9	2/9	7/9
	10× 0·2 ml.	4/9			3/9
	LD_{50}	6·4×0·2 ml.	Undil. 0·22 ml.	Undil. 0·26 ml.	5·2×0·2 ml.
	Sens. ratio		−7·1	−8·3	−1·2

* Number of injections of specific treatment.
† Number of deaths/total number.
‡ Number of intermediate injections of saline placebo.
§ The LD_{50} ratio of experimental group : control group, the figure of the latter being taken as unity. Plus sign means more sensitive, and minus sign less sensitive, than controls.

majority of the latter cases (experiments 2, 3, 4) the sheep rbc group was about 2–3-fold less resistant than the tetanus toxoid group, and only in experiment 5 did the sensitivity level of the 2 groups become approximately identical.

Experiment series II.—In this series the development of serum hypersensitivity in guinea-pigs which had been pretreated with diphtheria toxoid and killed typhoid bacilli was investigated. In one experiment the LD_{50} was measured, while in the other 3 experiments the sensitivity of the animals was tested by single point assay with 0·2 ml. of 2 × bovine serum. The results of these experiments are recorded in Table II.

Table II shows that the diphtheria toxoid-treated groups were consistently more resistant to serum shock than the control groups which received physiological saline as pretreatment. The degree of resistance is not impressive, the toxoid-treated groups being approximately 2-fold more resistant than the control groups in either LD_{50} measurement or single point assay with 0·2 ml. of 2 × serum. In groups treated with *Salm. typhi* vaccine no interference with serum hypersensitivity was demonstrable when the number of pretreatment did not exceed

TABLE II.—*Results of Serum Challenge of Guinea-pigs Pretreated with Diphtheria Toxoid and Killed Typhoid Bacilli*

Exp. No.	Challenge dose, LD_{50} and sensitivity ratio	Saline control	Diphtheria toxoid	Typhoid bacilli
6	2× 0·2 ml.	*2* 14/20	*2* 8/19	*2* 15/19
	Sens. ratio		−1·7	+1·1
7	Undil. 0·5 ml.	*3*	*2* 7/7	*3*
	2× 0·2 ml.	5/6	(*1*) 3/6	5/6
	10× 0·2 ml.	2/8	1/6	2/6
	50× 0·2 ml.	1/6		0/5
	LD_{50}	5·8×0·2 ml.	2·6×0·2 ml.	5·6×0·2 ml.
	Sens. ratio		−2·2	1
8	2× 0·2 ml.	*4* 16/20	*2* 8/20	*4* 12/20
	Sens. ratio		(*2*) −2·0	−1·3
9	2× 0·2 ml.	*4* 18/19	*2* 7/18	*4* 15/19
	Sens. ratio		(*2*) −2·4	−1·2

See footnote to Table I. Sensitivity ratios are ratio figures for LD_{50} or 2 × serum, as the case may be.

3 injections. With 4 injections of killed bacilli the serum hypersensitivity was slightly interfered with, but the degree of interference was even weaker than the diphtheria toxoid treated groups.

A rough comparison of the results recorded in Tables I and II can be made by pooling the mortality figures at the challenge dose of 2 × serum. The mortality figures of both experimental and control groups are first obtained, then the sensitivity ratios calculated and arranged in order of decrement. The figures are listed in Table III.

TABLE III.—*Serum Sensitivity Ratios of Experimental Groups Constructed by Pooling the Results of Challenge with 0·2 ml. of 2 × Serum*

	Tetanus toxoid	Sheep rbc	Diphtheria toxoid	Sheep rbc	Typhoid bacilli	Pertussis vaccine
No. of injection	2	4	2	3	4	4
Mortality of experimental groups	11/42 (26·2)*	7/17 (41·2)	26/63 (41·3)	8/12 (66·6)	27/39 (69·3)	12/17 (70·6)
Mortality of saline control groups	37/43 (86·1)	15/18 (83·3)	53/65 (81·5)	15/16 (93·8)	34/39 (87·2)	15/18 (83·3)
Sensitivity ratio	−3·28	−2·02	−1·97	−1·41	−1·26	−1·18

* Figures in parentheses indicate mortality per cent.

Figures obtained by single point assay as recorded in Table III give smaller difference of serum hypersensitivity than that obtained by LD_{50} determination. Thus in the 5 experiments in Table I the tetanus toxoid groups are at least 4-fold more resistant than the control groups in terms of LD_{50}, while the mean difference between the 2 groups is only 3·28-fold when assayed by the single dose of 2 × serum. It may be argued that too much confidence could not be placed on the exact order of serum sensitivity of the different groups as arranged in Table III, since the groups differ in sample size and in the number and time of experiment.

However, it is conceivable that the experimental figures probably would not deviate seriously from the true values, because the sensitivity ratios are constructed on the basis of the mortality figures of the control groups which were fairly constant throughout the experiment. Furthermore, fluctuation of sensitivity in any particular experiment caused by the interaction of environmental factors is likely to affect both experimental and control animals, so that the relative sensitivity ratio may be assumed not to be seriously affected.

γ-globulin determination.—If we accept the conventional view that serum anaphylaxis in guinea-pigs is an attribute of antibodies affixed to tissue cells (Burdon, 1956), and that the amount of such antibodies determines the intensity of serum sensitivity in this species, then the different degrees of sensitivity displayed by the various experimental groups mentioned above may be explained by the different intensities of interference the various antigens exerted on the development of sessile antibodies to bovine serum. Along this line of reasoning, it will be of interest to examine the intensity of immune responses induced by the injection of various antigens employed.

The comparison of intensity of immune response between different groups can best be performed by measuring the antibody nitrogen. This approach is practically impossible in this study, however, in view of the complex antigenic character of some of the antigens used, such as sheep rbc, pertussis and typhoid bacilli. Accordingly, we had recourse to the use of serum γ-globulin concentration as a working criterion for judging the intensity of immune responses.

For this purpose groups of 10 animals were immunized between days 1–20 with 2 doses of tetanus or diphtheria toxoid (saline placebo being used for the 2 intermediate injections), 4 doses of sheep rbc, killed typhoid bacilli or pertussis vaccine respectively. Another group which received 4 injections of saline was left as a control. On the 41st day the animals were bled from the heart, the sera separated and their γ-globulin content determined. The results are given in Table IV.

TABLE IV.—*Mean Serum γ-globulin Concentration in Immunized and Control Guinea Pigs (mg./100 ml.)*

	Pertussis vaccine	Tetanus toxoid	Sheep rbc	Diphtheria toxoid	Typhoid bacilli	Saline control
No. of injections	4	2	4	2	4	4
No. of animals	10	9	10	10	10	10
Mean ± s.e.	703±0·03	580±0·04	573±0·03	561±0·04	552±0·04	519±0·03

It is clear from Table IV that antigenic stimuli invariably caused demonstrable increase in the concentration of serum γ-globulin. With the specified dosage and number of injections employed, the pertussis vaccine caused the greatest increase (184 mg./100 ml. serum) in serum γ-globulin and the killed typhoid bacilli the smallest (33 mg./100 ml. serum). The differences between tetanus toxoid, sheep rbc, diphtheria toxoid and killed typhoid bacilli are in the order of about 10 mg. respectively, but the difference between pertussis vaccine and tetanus toxoid, placed second in the series, amounts to as much as 123 mg.

The serum γ-globulin increase in the order of tetanus toxoid, sheep rbc, diphtheria toxoid and killed typhoid bacilli agrees with the sensitivity ratio order as listed in Table III. The fact remains, however, that the pertussis vaccine

induced the greatest increase in serum γ-globulin in this series and yet exerted no interference on the rate of serum anaphylaxis.

DISCUSSION

It is well known that in most species the bulk of circulating antibody molecules are associated with the γ-fraction of serum proteins and that antibody formation usually results in the increase of concentration of serum γ-globulin (Gutman, 1948). One may as well be conservative as to the validity of measuring intensity of immune response by means of serum γ-globulin quantitation, since the presence of antigen may induce, besides antibody formation, increase in nonspecific globulins (Boyd and Bernard, 1937). However, in the absence of existing evidence which points to the disproportional increase of nonspecific globulins induced by antigens employed in this study, and there is no solid theoretical reason *a priori* to suggest that such evidence may appear, the inadequacy of using γ-globulin quantitation as a working criterion for judging the relative intensity of immune responses can not be over-emphasized.

The work of Adler (1957) showed that antigens may differ in their ability to interfere with a specified heterologous immune response. In the series of antigens tested by us in this experiment, the pertussis vaccine is unique in that it apparently induced the greatest immune response as it caused the greatest increase in serum γ-globulin concentration, yet it hardly interfered with the serum hypersensitivity under the conditions of our experiment. It appears therefore that the quality of an antigen may determine its ability to interfere with heterologous antibody formation. Presumably this may be one of the reasons why some earlier workers failed to demonstrate the existence of the " crowding-out " phenomenon despite the concurrent injection of multiple antigens (Topley and Wilson, 1946).

Exactly what antigen quality determines its ability to cause heterologous interference can not be answered with certainty at present. In this connection it is interesting to note that according to Barr and Llewellyn-Jones (1953), batches of typhoid-paratyphoid vaccine may differ in their ability to interfere with tetanus antitoxin formation in the guinea-pig. It is well known that pertussis vaccine has an adjuvant effect on the development of serum anaphylaxis in the mouse (Malkiel and Hargis, 1952). Whether this property of pertussis vaccine has anything to do with the unique behavior shown in this study is under investigation.

Chen *et al.* (1956) showed that in primary diphtheria-pertussis-tetanus combined vaccination in children, the intensity of heterologous interference of pertussis and tetanus responses was significantly different between children with a pre-existing diphtheria antitoxin level above 0·01 unit/ml. and those with a level below this figure. Adler (1957) showed that in concurrent immunization of guinea-pigs with washed specific precipitate and haemocyanin, the degree of interference with antibody formation against rabbit immune globulin was roughly proportional to the concentration of haemocyanin antibody produced. In the present study the increase in serum γ-globulin content is taken as the common criterion for judging the intensity of immune responses caused by different antigens, and the available data show that γ-globulin increase in the order of tetanus toxoid, sheep rbc, diphtheria toxoid and killed typhoid bacilli agrees with the interference order of these antigens. The evidence thus far, therefore, is in

favour of a sequential quantitative relationship between the intensity of antibody response against the competing antigen and the degree of heterologous interference. The report of Abramoff and Walfe (1956) is very interesting in that the simultaneous injection of bovine haemoglobin suppresses markedly the antibody response to bovine serum albumin even though there is no detectable antibody formation to the competing haemoglobin, but we hesitate to accept their interpretation. Failure to demonstrate antibodies with the conventional serological technique does not necessarily exclude their existence, and we feel that such negative findings should be confirmed by some other techniques such as quantitative serum γ-globulin estimation.

The interfering action of tetanus toxoid shown in this study is moderately strong at best, and it remains to be seen whether it can be enhanced. However, the moderate difference of LD_{50} between tetanus toxoid and control groups may reflect a tremendous difference in the amount of sessile antibodies between the groups, if inferences can be drawn from passive transfer experiments (Kabat, Coffin and Smith, 1947). That serum anaphylaxis can be suppressed measurably by harmless procedures as shown in this study, plus the encouraging result of Rh countersensitization by means of " crowding-out " (Unger, 1950; Shanaphy, 1952), leads one to speculate on the possibility of applying the principle of heterologous interference in the treatment of other allergic conditions.

The mean value of 519 mg. γ-globulin per 100 ml. serum and the relative composition of 10·6 per cent γ-globulin in total serum protein of the normal guinea-pig obtained by us in this study are compatible with those reported by Hudgins *et al.* (1956) and Moore (1945). However, our figures are greater than those of Deutsch and Goodloe (1945), but definitely smaller than those reported by Svensson (1946) and Weimer *et al.* (1955). Probably different strains of guinea-pigs and dissimilar diet and care might be the source of variation, as Moore has shown that the serum γ-globulin content may differ as much as 3–5 times between strains of rats.

SUMMARY

Guinea-pigs were pretreated with conventional antigens and then sensitized and challenged with bovine serum. Animals similarly treated but without the serum sensitization were bled for titration of serum γ-globulin. With specified dosage and number of injections of the antigens tested, the pertussis vaccine induced the greatest increase in serum γ-globulin concentration and yet exerted hardly any interference on the development of serum anaphylaxis. The order of intensity of interference with serum hypersensitivity in animals treated with tetanus toxoid, sheep red blood corpuscles, diphtheria toxoid and killed typhoid bacilli agrees with the order of increase in serum γ-globulin concentration these antigens induced.

The data suggest that antigens may differ qualitatively in their ability to interfere with the antibody formation against a specified heterologous antigen. Where interference can be demonstrated, there may exist a quantitative relationship between the intensity of antibody formation against the competing antigen and the degree of interference with antibody response against the heterologous antigen.

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OBSERVATIONS ON THE PATHOGENESIS OF FIBROSIS IN RATS ON A PROTEIN DEFICIENT DIET

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In biopsy specimens of the liver of patients with kwashiorkor, the presence of small collections of inflammatory and reticulo-endothelial cells surrounding degenerating liver cells has been noted. Although in uncomplicated and usually mild cases of kwashiorkor such as are seen in Jamaica, such cellular foci are not present in any quantity, yet they do occur regularly (Bras, 1955). They have been interpreted as representing cellular reaction to hepatocellular degeneration and/or necrosis. Following treatment, restitution is complete and there is no subsequent fibrosis (Back and Bras, unpublished).

We were struck by the presence of cellular foci similar to, but more abundant than, those which occur in the livers of patients with kwashiorkor during the examinations of the livers of many hundreds of rats, fed for various periods on one of the following experimental cirrhosis producing diets.

A.—Vitamin-free casein 8, lard (or Crisco) 38, cod liver oil 2, Sucrose 48, and U.S.P. salt-mixture 4.

B.—Vitamin-free casein 8, lard (or Crisco) 6, cod liver oil 2, Sucrose 80 and U.S.P., salt-mixture 4.

C.—Extracted peanut meal 30, casein 6, lard 18, cod liver oil 2, Sucrose 40 and U.S.P. salt-mixture 4.

D.—Extracted peanut meal 30, casein 6, Crisco 18, Sucrose 42, U.S.P. salt-mixture 4 and 3 drops perc. oil/wk.

E.—Extracted peanut meal 30, lard 18, cod liver oil 2, Sucrose 46 and U.S.P. salt-mixture 4.

The quantities in all the diets are in parts per hundred unless otherwise stated.

These cellular foci were noted mainly in those areas of the liver where fatty change had developed, *i.e.*, the non-portal regions (Fig. 1, 1*a*, 2) and almost exclusively in sites where fatty change existed, although this was not necessarily severe. They were more pronounced in the early stage of hepatic injury (50 days) than after fully developed general fibrosis.

This cellular infiltration was present (*a*) surrounding fatty liver cells, or, around globules of fat in disintegrating liver cells (Fig. 3, 4); (*b*) occasionally surrounding liver cells with eosinophilic degeneration of the cytoplasm, without apparent fatty change; (*c*) also in what appeared to be inter-cellular tissue, no liver cells being demonstrable in the areas involved (Fig. 5). This may mean,

* Supported by grants of the Josiah Macy, Jr. Foundation and the Nutrition Foundation to one of us (P.G.).

however, that this space had been occupied by liver cells which had disintegrated completely.

The foci of cellular infiltration were much less pronounced in the livers of animals fed the cirrhosis-producing diet with supplement of methionine, 50 mg. daily, which resulted in the deposit of not more than small amounts of fat in the liver cells. When 20 per cent of casein was present in the diet, no fatty change developed in the liver and foci of cellular infiltration did not occur.

Ceroid deposit was present in these areas of cellular infiltration, but in livers with little or no ceroid (Crisco as the source of fat in the diet) similar cellular foci occurred. Also, in well established dietary cirrhosis, the fibrous bands contained an abundance of ceroid but little or no cellular infiltrate. The presence of ceroid *per se* then cannot account for the cellular foci.

Apart from the well known fatty change, the liver cells showed such changes as mitoses, hyperchromatism and polyploidy. The portal triads sometimes displayed an increase in cells, especially around the bile ducts. Some of these cells were polymorphonuclears, but most of the cells were lymphocytes or reticulum cells and there was some proliferation of ductular lining epithelial cells. Liver cells, some well preserved, and others degenerating, necrotic, or containing fat globules, were enmeshed in the areas of fibrosis (Fig. 6).

Fatty cysts, as described by Hartroft (1950), were not much in evidence in our rats.

It appears, then, that in the livers of rats with this dietary deficiency changes other than fatty metamorphosis, occur which evoke cellular infiltrate. From the localization of the infiltrate, this additional factor seemed mostly to be localised around cells showing fatty change, but occurred occasionally away from the obviously fatty cells and was not always present in the precise centre of the lobules where the fatty change was most pronounced. In areas where the cellular foci were present, fibrosis developed, which coincided with the site of disappearance of liver cells.

It seems, that the factor which evokes the cellular infiltration is a slowly developing, piecemeal necrosis of the liver cells, frequently but not necessarily preceded by fatty metamorphosis. We believe, therefore, that parenchymal degeneration and cellular infiltration play a part in the development of cirrhosis due to dietary deficiency in rats, the fibrosis being the end result of a continuous gradual necrosis of the liver cells initially evidenced by cellular reaction surrounding these cells, and being either due to sustained fatty change or to the specific deficiency.

DISCUSSION

There is no doubt that degeneration and necrosis of liver cells have been observed by various investigators of cirrhosis. Some investigators of experimental dietary cirrhosis have incriminated the sustained fatty change in the liver cells as being responsible for the subsequent fibrosis (Daft, Sebrell and Lillie, 1942; Himsworth and Glyn, 1944).

Hartroft and Ridout (1951) considered the collapse of fatty cysts as the main cause of stromal condensation which results in the formation of fibrous septa in the liver of rats fed on a diet deficient in choline and methionine, but György and Goldblatt (1949) noted in the livers of such experimental animals the common

occurrence of degenerated or necrotic hepatic cells, in addition to the fatty change, preceding the fibrosis.

Koch-Weser *et al.* (1953) observed necrosis of liver cells, with accumulation of replacement cells, in rats on a high fat low protein diet. Similar foci were also seen by Nino-Herrera *et al.* (1954) in rats fed on a diet containing 9 per cent casein, with a choline supplement. Apparently the protein deficient diet induced cellular damage not prevented by a supplement of choline.

Popper and Schaffner (1957), in a study of human material (presumably the fatty livers of alcoholics) clearly described and gave pictures of fatty change, hepatocellular damage and necrosis, with the presence of reactive cellular infiltration.

So far as we are aware, most investigators, either implicitly or explicitly, have accepted the fact that sustained fatty change, perhaps supported by anoxemia in the central lobular areas (Himsworth, 1950), causes death of liver cells, while Hartroft (1950) demonstrated rupture and collapse of hepatic fatty cysts. Fatty cysts did occur in our material, but they were not striking, and their collapse did not seem to account in any great part for the development of the fibrous septa. Differences in the diet employed by Hartroft and by us may account for this.

We are impressed by the apparent importance of degeneration and necrosis of liver cells and the cellular reaction they evoke, both in experimental dietary cirrhosis and in human cases of protein deficiency. We incline, therefore, to the view that such degeneration and the inflammatory infiltrate are important pathogenetic factors in the development of fibrosing liver disease. They apparently may be caused by choline and methionine (protein) deficiency, while toxic or infectious noxious agents may enhance their development and increase their number. When the cellular foci are present in sufficient quantity they are indicative of liver disease progressing toward fibrosis.

SUMMARY

White, male, Sprague-Dawley rats on a protein deficient diet developed fatty change in parenchymal cells of the liver, followed by fibrosis. Under these circumstances, however, the fibrosis seemed to occur as a result of a continuous gradual necrosis of liver cells rather than through formation of fatty cysts.

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EXPLANATION OF PLATES.

FIG. 1.—Low power view of liver lobule with portal triad (P) and central vein (C.V.). Note cellular foci towards the lobular centre. H.E. Section ×125.

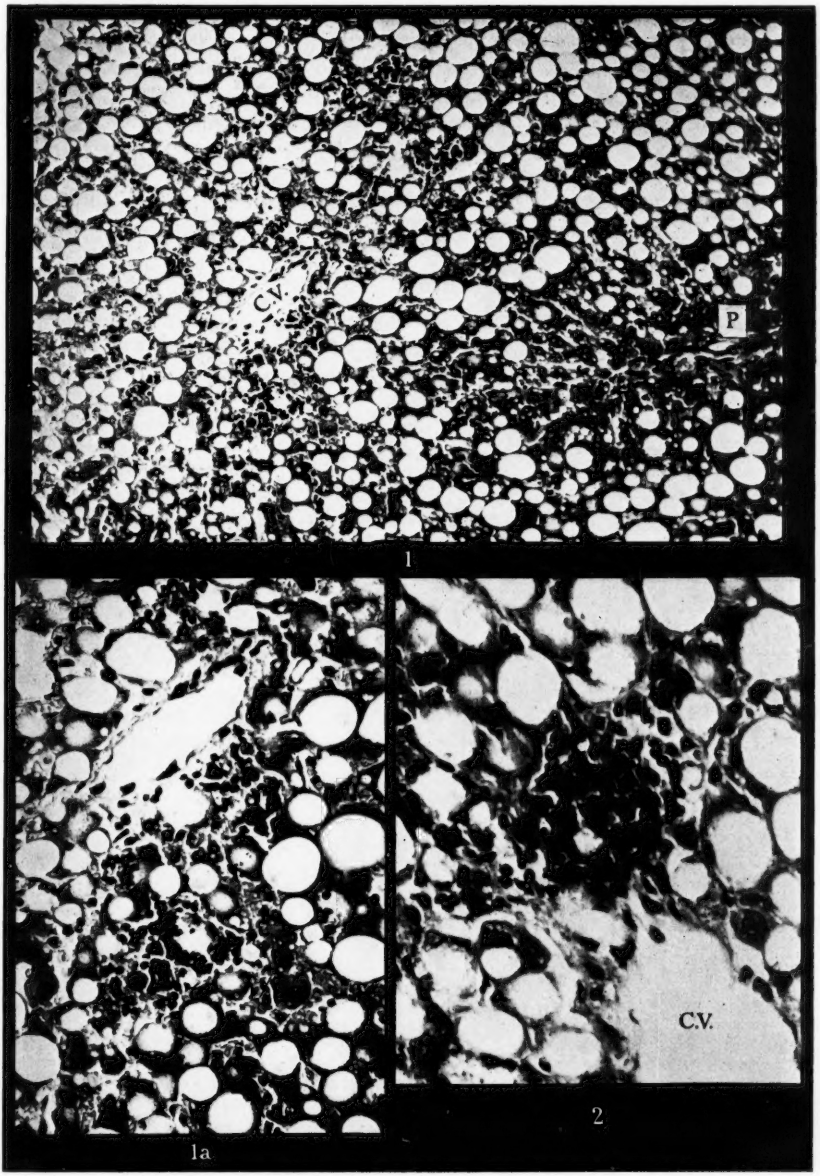
FIG. 1A.—Close-up of centrolobular area in Fig. 1. ×243.

FIG. 2.—Focus near central vein (C.V.). H.E. ×300.

FIGS. 3 and 4.—Focus surrounding fatty liver cell (fat globules). H.E. ×427.

FIG. 5.—Focus located " interstitially ". H.E. ×427.

FIG. 6.—Centrolobular area showing fibrosis. H.E. ×127.



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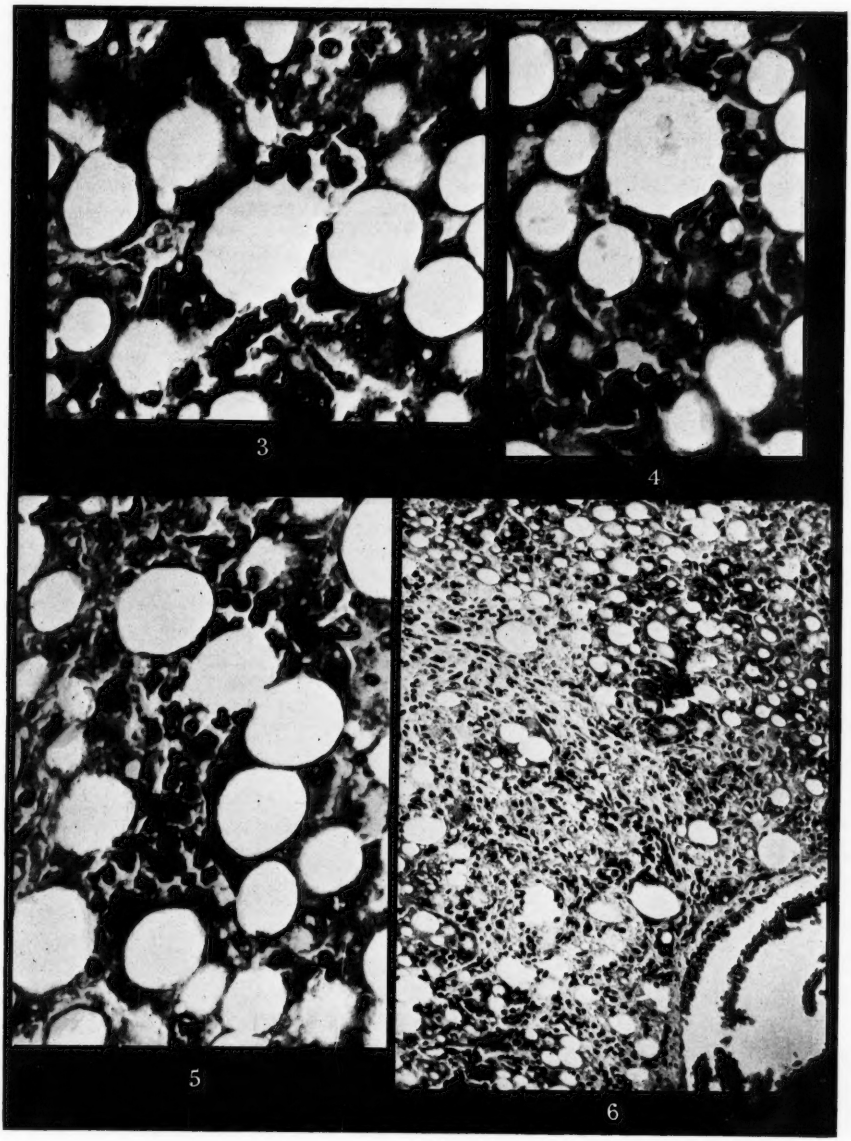

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SOME HISTOLOGICAL ASPECTS OF CONNECTIVE TISSUE METABOLISM IN ACUTE SCORBUTUS

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A FLORID granulomatous reaction resulting from the subcutaneous injection into guinea-pigs of carrageenin, a sulphated polygalactose extracted from Irish moss, was described by Robertson and Schwartz (1953). Their studies of connective tissue metabolism in the carrageenin granuloma have been extended from both the biochemical and histological aspects (Jackson, 1957 ; Slack, 1957 ; Williams, 1957). More recently the latter workers have applied this experimental model to scorbutic guinea-pigs with a view to studying the role of vitamin C in connective tissue metabolism (Slack, 1958). This paper deals with the histological aspects of the connective tissue reaction to subcutaneous and intradermal injections of carrageenin in guinea-pigs suffering from acute scurvy.

MATERIALS AND METHODS

Albino guinea-pigs of both sexes, of weights ranging from 280–350 g., were fed on a scorbutogenic diet (Kodicek and Loewi, 1955) throughout the experiment. After 7 days on this diet to deplete their natural resources of vitamin C, the animals were divided into 2 groups and treated as follows :

Group I (24 *animals*).—Under open ether anaesthesia, each animal was injected subcutaneously in the lower abdominal wall with 5 ml. of a 1 per cent solution of carrageenin in 0·9 per cent NaCl. A linear wound, 1 cm. long and involving the whole skin thickness, was made in the left buttock of each animal.

Group II (20 *animals*).—Under ether anaesthesia, 2 sq. in. of the skin in the region of the nape of neck were shaved and with an intradermal needle 0·5 ml. of a 1 per cent solution of carrageenin in 0·9 per cent NaCl was injected intradermally as five equal doses at separate sites in linear arrangement. Equal volumes of 0·9 per cent NaCl were injected parallel to the carrageenin injections as carrageenin controls. Buttock wounds were inflicted as in Group I animals.

Control animals.—Carrageenin controls for Group I consisted of 5 guinea-pigs fed the scorbutogenic diet and injected subcutaneously in the belly wall with 5 ml. of 0·9 per cent NaCl. Another 5 guinea-pigs fed the scorbutogenic diet but supplemented with daily fresh greens and on alternate days by 50 mg. of ascorbic acid in sucrose by mouth, served as scurvy controls ; they received subcutaneous carrageenin injections similar to the test animals. Similar control groups of 5 animals were used for the intradermal study.

Behaviour of animals.—After institution of the scorbutogenic diet the growth curves of the animals rose steadily for 10 days, remained stationary for a further 5–6 days and then fell steadily until time of death or killing. The maximal survival period in either group was 25 days. Within this period and mainly in its later stages, nine animals of Group I and 5 of Group II died from acute scurvy and pulmonary haemorrhages and were discarded. The loss of weight affecting the surviving animals in the later stages coincided with a refusal to eat the diet, and hence starvation as well as vitamin C deficiency accounted for the weight loss. An arbitrary period of 17 days after commencing the scorbutogenic diet (*i.e.* 10 days after injection) was therefore taken as the period in which the tissue changes could be attributed to the effects of vitamin C deficiency alone.

In contrast to the test animals, the non-scorbutic (control) animals continued to gain weight steadily up to the day of killing.

Histological material.—The 15 surviving animals in each group were killed in groups of 3 on the 3rd, 5th, 10th, 14th and 18th days after injection and wounding, although the changes observed in the 14th and 18th day specimens were, for the reasons just given, not regarded as exclusively due to scurvy.

From Group I animals, whole thickness slices of the belly wall were taken for histology; from Group II animals the whole skin thickness of the injection area was excised, pinned flat on shallow cork trays, allowed to harden at —10° and cut into parallel strips approximately 2 mm. in thickness. To assess the degree of scurvy affecting the animals wounds were also taken for histological examination and assays of ascorbic acid content were made of adrenal glands by the method of Glick (1954) shortly after death.

Corresponding tissues were taken from control animals.

Tissues were fixed in either 10 per cent formol-saline, or Helly's fluid; for acid mucopolysaccharide fixation a solution of 0·4 per cent 5-aminoacridine hydrochloride in 50 per cent ethanol was found useful (Williams and Jackson, 1956). Tissues were examined mainly as paraffin sections stained with haematoxylin and eosin; Foot's silver impregnation method with Van Gieson counterstain, and Mallory's trichrome method were used for connective tissue fibres; the periodic acid-Schiff-haematoxylin (modified McManus, 1946) and toluidine blue (Grübler) stains were used for mucopolysaccharides. Numerous examinations of fixed material were also made under polarized light.

RESULTS

Group I: Subcutaneous injection of carrageenin

Within 24 hr. the carrageenin diffuses to a varying extent over the abdominal wall as a gelatinous layer. By the 3rd day, the carrageenin is invaded by scanty polymorphs and macrophages in contrast to the more intense inflammatory reaction at this stage in the non-scorbutic control animals (Fig. 1). By the 5th day the inflammatory reaction is intensified and numerous histiocytes and fibroblasts begin to invade the margins of the carrageenin pool from both the overlying dermis and the underlying muscle layer. Most of the fibroblasts are plump cells, some with small hyperchromatic nuclei, others with larger, pale, vesicular nuclei and open chromatic networks. Their cytoplasm occasionally shows small vacuoles and PAS positive, non-metachromatic granules. The histiocytes are mainly spherical cells with nuclei displaced eccentrically by ingested carrageenin which stains metachromatically. Occasional multinucleated phagocytes are present.

The fibroblasts continue to proliferate but retain their immature form. Thus by the 10th day a broad cellular zone encircles the carrageenin pool but lacks the regularity and compactness of normal granulation tissue; capillaries are few and poorly formed and intercellular connective tissue fibres fail to develop (Fig. 2) although occasional irregular argyrophil deposits are present between the cells. Haemorrhages are fairly common in the cellular zone. The overlying dermis shows increased cellularity due to infiltrating macrophages and locally stimulated fibroblasts but there is minimal alteration of its preformed collagen fibres.

By the 14th day, much of the carrageenin has been ingested by histiocytes and blood leucocytes or absorbed and although fibroblast proliferation continues there is no formation of new connective tissue fibres. Thereafter, presumably due to death and resorption of macrophages and the continued uptake of extracellular carrageenin into the lymphatics, the cellular mass becomes smaller, the ultimate degree of resorption being determined by the survival period of the

animal. In the present experiments none of the animals survived after the 18th day following injection.

In contrast, the non-scorbutic animals by the 5th day show a more vigorous granulomatous reaction with formation of new connective tissue fibres, abundant mature fibroblasts, patent capillaries and fewer haemorrhages, and showed a normal granulomatous response throughout the remainder of the experimental period.

Group II : Intradermal injections of carrageenin

The cellular reaction in the early stages closely parallels that seen in the subcutaneous granuloma, namely a mild leucocytic infiltration of the injected area after 3 days, followed in 5 days by a more intense macrophage response and the local proliferation of immature fibroblasts. This cellular activity separates the dermal collagen fibre bundles but does little to alter their physical appearance or their tinctorial reaction to the connective tissue stains. For instance, after 10 days, when stained by the silver impregnation/Van Gieson combination the fibres take up the acid fuchsin and show limited argyrophilia (Fig. 3). After 14 days the preformed collagen fibres are still well preserved and after 18 days the only significant change is the formation of vacuolated spaces between the fibre bundles in the dermis of 2 of the animals ; this is accompanied by a simultaneous decrease in dermal cellularity.

The non-scorbutic animals show a comparable cellularity of the dermis by the 5th day although the fibroblasts are of mature type. In contrast however there is progressive breakdown of dermal collagen into thinner argyrophil fibres, a reaction which is well advanced by the 10th day (Fig. 4) and progresses thereafter until the time of killing.

Wounds, joints and adrenal glands.—In the non-scorbutic animals the buttock wounds heal normally with organization of the wound exudate, epithelization of the surface and reconstitution of the dermis by scar tissue (Fig. 5). Knee

EXPLATION OF PLATE.

FIG. 1.—(*a*) Non-scorbutic (control) guinea-pig. (*b*) Scorbutic guinea-pig. Sections of abdominal wall on third day after subcutaneous injection of carrageenin. Note the delay in the inflammatory response in scorbutic animal. Haemalum and Eosin, ×65.

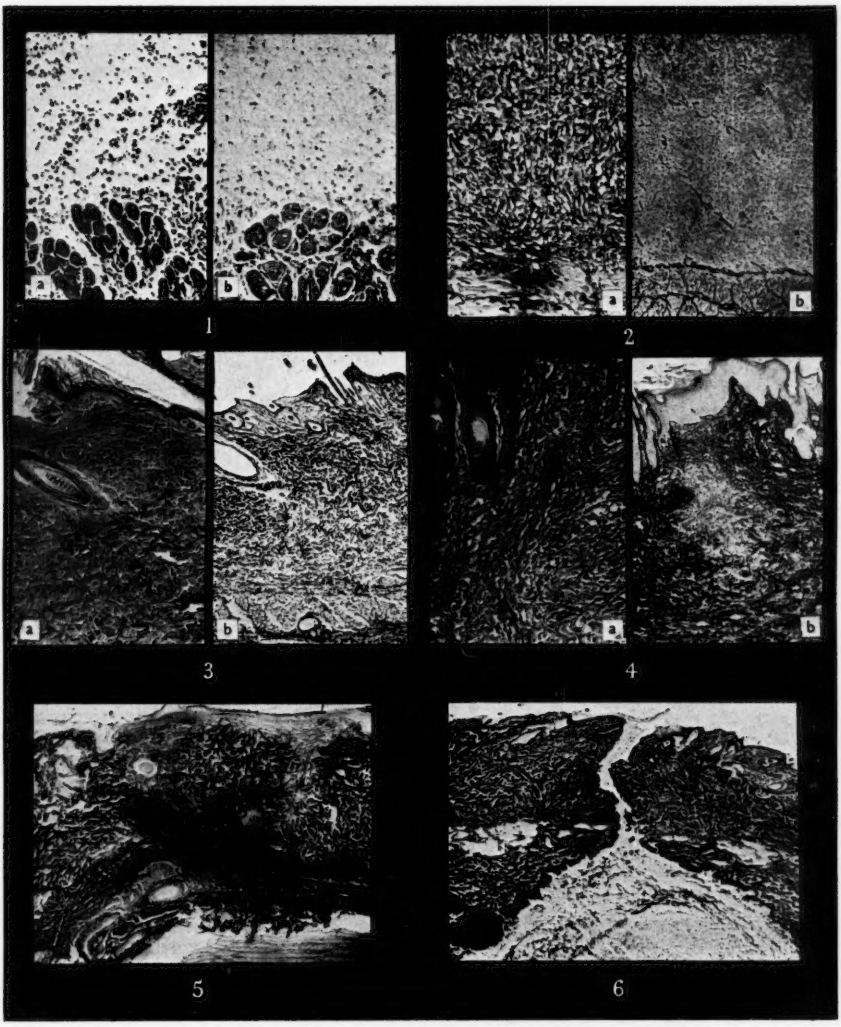
FIG. 2.—Scorbutic guinea-pig. Serial sections of edge of carrageenin granuloma on 10th day after injection, showing (*a*) proliferation of fibroblasts and histiocytes but (*b*) absence of normal connective tissue fibres. (*a*) Haemalum and Eosin. (*b*) Foot's siver impregnation/Van Gieson. ×55.

FIG. 3.—Scorbutic guinea-pig. Serial sections of dorsal skin on 10th day after intradermal injection of carrageenin showing (*a*) increased cellularity of dermis and (*b*) separation, but minimal breakdown of collagen fibre bundles. (*a*) Haemalum and Eosin, ×65. (*b*) Foot's silver impregnation/Van Gieson, ×30.

FIG. 4.—Non-scorbutic guinea-pig. Serial sections of dorsal skin 10 days after intradermal injection of carrageenin showing (*a*) proliferation of dermal fibroblasts and macrophages and (*b*) breakdown of collagen into irregular argyrophilic fibres. (*a*) Haemalum and Eosin, ×65. (*b*) Foot's silver impregnation/Van Gieson, ×30.

FIG. 5.—Non-scorbutic (control) guinea-pig. Buttock wound on 10th day showing advanced state of healing with abundant new collagen fibre formation. Foot's silver impregnation/Van Gieson, ×30.

FIG. 6.—Scorbutic guinea-pig. Buttock wound on the 10th day. There is re-epithelization of surface but failure of connective tissue regeneration in wound exudate. Foot's silver impregnation/Van Gieson, ×30.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joints show a normal growth pattern at the epiphyseal cartilage and normal articular surfaces. The incisor teeth are normal.

In contrast, in animals fed the scorbutogenic diet, the wounds fail to heal; invasion of the wound exudate by immature fibroblasts is rapidly followed by the appearance of excessive non-metachromatic ground substance and occasional extracellular deposits of argyrophil material but there is total absence of normal fibrillar collagen (Fig. 6). Epithelization of the wound surface is not impaired. The knee joints show haemorrhages into the joint cavities and beneath the periosteum with varying degrees of cellular invasion. In the early stages the bone marrow in the region of the epiphysis is haemorrhagic and oedematous; later, the bone shows disordered trabeculae, failure of ossification of calcified cartilage and varying degrees of fibroblastic proliferation in the marrow spaces and beneath the periosteum.

Ascorbic acid assays of the adrenals in the scorbutic group showed levels varying from 0·06–0·18 mg./g. wet weight; those of the controls averaged 1·6 mg./g. wet weight.

DISCUSSION

The general picture of human and experimental scurvy has been well characterized (for reviews see Bourne, 1942 and 1957; Follis, 1948; and Wolbach, 1953). It has also been shown (Pirani, Bly and Sutherland, 1950; Kodicek, 1951) that the skeletal changes vary considerably according to the degree of scurvy. The age of the animals is also important; total deprivation of vitamin C producing a more fulminating disease process than partial deficiency and younger animals suffering more acutely than their adult counterparts. Within narrower limits, there may also be variation between animals of the same age subjected to strictly similar regimes. It is important therefore in investigations of this nature that the experimental conditions should be clearly defined.

In the present investigations total deprivation of vitamin C produced signs of acute scurvy within 7 days, the disease running a rapid course and producing progressive weight loss after approximately 14 days from commencing the diet. In a comparable series of scorbutic animals it was found that if ascorbic acid were given (50 mg. by mouth on alternate days within a few days of the onset of loss of weight) the animals showed a rapid improvement in their general condition and regained a positive weight balance. If totally scorbutic conditions were maintained for a longer period, the administration of vitamin C had no such beneficial effects, the animals continuing to lose weight and dying within 25 days of commencing the scurvy diet; these animals frequently showed adrenal necrosis and pulmonary haemorrhages. Fully grown adult guinea-pigs survived the same scorbutogenic regime for a test period of 3 months, producing lesions of a more chronic nature presumably due to their decreased metabolic requirements.

The subcutaneous granulomata (Group I) showed the characteristic histological features of scorbutic tissue, namely proliferation of immature fibroblasts, deficient capillaries with frequent haemorrhages and absence of mature collagen fibres. Occasional irregular argyrophil fibrillar deposits were seen in both the scorbutic and control granulomata and hence could not be conclusively attributed to the scorbutic state although their presence in the scorbutic, and absence from the control wounds made this feasible. Similar bloated argyrophil fibres were de-

scribed in scorbutic wounds by Hunt (1941) and Danielli, Fell and Kodicek (1945).

Despite the absence of histologically normal collagen fibres, Jackson (personal communication) was able to demonstrate neutral salt soluble, acid soluble and insoluble collagen fractions in carrageenin granulomata in a comparable series of scorbutic guinea-pigs although in considerably lower concentration than in granulomata in normal animals. Some of the extractable collagen may have resulted from contamination by surrounding collagenous tissue during the dissection of the granulomata from the abdominal wall. Alternatively it may have been derived from the breakdown and diffusion into the granulomata of preformed surrounding collagen. However, this seems less likely in view of the present experiments in which collagen breakdown was not a prominent feature under scorbutic conditions. Another explanation might be that in scurvy, fibroblasts elaborate a collagenous protein or "pre-collagen" which, in the absence of vitamin C is incapable of forming mature fibres, a concept first put forward by Wolbach and Howe (1926) in their "gelation" theory of fibrogenesis. More recently it has been suggested (Robertson and Schwartz, 1953) that vitamin C may influence the chemical composition in this collagen protein, in that it may be necessary for the synthesis of hydroxyproline or its incorporation into the collagen macromolecular. Gould and Woessner (1957), from their investigations of scorbutic wounds in guinea-pigs, have suggested that vitamin C promotes the synthesis of hydroxyproline from a protein pool rich in proline and glycine, thereby enabling fibrillar collagen to form. Robertson has now produced evidence to show that his original hypothesis is no longer tenable. The material which he suggested might be a protein-rich precursor has been shown to be a mixture of normal gelatin and a protein fraction with a proline concentration considerably lower than that of normal collagen (Jackson, personal communication).

Biochemical investigations of the carrageenin granulomata in scorbutus have also shown that there is a substantial (approximately 5-fold) increase in mucopolysaccharide content as compared to that of granulomata in normal animals. Robertson and Hinds (1956) claim to have isolated and identified the predominating polysaccharide as hyaluronic acid, probably in a highly polymerized form. Slack (1958) describes this mucopolysaccharide as having properties similar to hyaluronic acid and draws attention to a decreased production of chondroitin sulphate. Since neither of these polysaccharides is Schiff-positive following periodate oxidation and since both carrageenin and chondroitin sulphate react metachromatically to toluidine blue, it is not surprising that histological examination is of limited value in assessing these ground substance changes in this particular biological situation.

In Group II animals the main point of interest was the reaction of the dermal collagen to the carrageenin injections. The dermal fibre bundles, though separated by the injection material and infiltrated by the proliferating connective tissue cells, showed only limited histological evidence of fibre breakdown. In contrast, the non-scorbutic control animals in common with a series previously described by Williams (1957) showed progressive degradation of dermal collagen in the injection area. It was not possible to establish a suitable end point for quantitative comparison between these control and scorbutic skin lesions on account of the short duration of the experiment permitted by dietary conditions. Qualitatively, however, the differences appeared sufficiently significant and consistent

to indicate that in acute scorbutus the dermal collagen fibres are less susceptible to breakdown under the stimulus of carrageenin injection than those of animals on normal diet.

From the evidence available it would appear that *in vivo*, at least, absence of vitamin C arrests normal fibroblast development, interfers with the synthesis of normal collagen fibres, and alters the normal pattern of mucopolysaccharide production. Which of these 2 deranged metabolic processes is the more vital or whether both are equally so remains unknown. However, since the syntheses of protein and polysaccharide have totally different metabolic pathways it may be that vitamin C influences even more basic metabolic functions in the connective tissue cell. Furthermore, it would appear that the mechanisms involved in collagen fibre synthesis and breakdown are in some way related since both are inhibited by vitamin C deficiency.

SUMMARY

A histological study was made of the changes produced by subcutaneous and intradermal injections of carrageenin, a sulphated polygalactose of sea-weed origin, into guinea-pigs suffering from acute scorbutus.

Subcutaneous injections produce an acute inflammatory response which is delayed in scorbutic, as compared to the normal control animals. This is followed after 5 days by a granulomatous reaction in which histiocytes and fibroblasts predominate. The fibroblasts are of immature type, but their proliferative power is not impaired. By the 10th day, these cells form a broad zone around the injected carrageenin but there is no evidence of normal intercellular fibrogenesis, only occasional irregular argyrophil deposits being seen. After 14 days much of the carrageenin has been phagocytosed and thereafter the cellular mass decreases in size.

Following the intradermal injections, there is a transient leucocytic response followed by marked macrophage and fibroblastic reaction within the dermis. This produces from the 5th day, separation of the collagen fibre bundles but only slight evidence of collagen breakdown, in contrast to the control animals in which marked collagen degradation is present. These findings are discussed in relation to biochemical observations made in similar experiments.

The evidence indicates that connective tissue fibre synthesis and breakdown are interrelated since both are inhibited in the absence of vitamin C.

I am indebted to Drs. D. S. Jackson and H. G. B. Slack with whom I collaborated in this investigation. I also wish to acknowledge the helpful criticism of Professors J. H. Kellgren and A. C. P. Campbell and Dr. J. Ball in preparing the manuscript and the assistance of the laboratory technical staff with the histological preparations.

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HEPATIC LESIONS AND ASCITES IN EXPERIMENTAL LONG-TERM CONSTRICTION OF THE INFERIOR VENA CAVA IN THE RAT

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RICHARD LOWER (1631–91) was the first to report on ligation of the inferior vena cava above the liver in animals. He observed ascites in a dog which died a few hours after he performed the operation. Starling (1894) showed that the flow of lymph from the liver was increased following obstruction of the cava above it in the dog. There are now many reports of the long-term results of severe experimental constriction of the upper part of the inferior vena cava in this species (*e.g.* Zimmermann and Hillsman, 1930; Kershner, Hooton and Shearer, 1946; Volwiler, Grindlay and Bollman, 1950; Belli and Sprovieri, 1956; Davis, Pechet, Ball, Goodkind and Casper, 1957). When the constriction is sufficient to cause marked retrograde venous congestion and yet not severe enough to kill the animal, ascites is detectable within a few weeks. The effusion is copious and may comprise up to 35 per cent of the total body weight. After many months the ascites tends to remit spontaneously. It may be questioned to what extent the results of these experiments on dogs can be transposed to human conditions, for the hepatic-venous system of the dog is unique in the exceptional degree to which the muscle and the associated lymphatics are developed (Arey, 1941).

In structure and arrangement, the hepatic veins and lymphatics of the cat and of the rabbit are more like those of man. Bolton (1910) and later Barnard with him (1928) studied the ascites and other long-term effects of partial constriction of the inferior vena cava in the thorax of the cat. The ascites was less, only about 2 per cent of total body weight, and tended to remit sooner than that in dogs. Results similar to those in cats have been obtained in rabbits (Milnes, 1951; Kawano, 1957).

Since the structure of the hepatic veins and the arrangement of the lymphatics might be expected to influence the effects of caval constriction, it is valuable to study the experimental condition in an animal in which these are relatively simple. The rat fulfils the requirement, for the hepatic veins contain considerably less muscle than those of man and are little more than fibrous tubes; the liver capsule is thin and practically free of lymphatics. These differences between the rat liver and the human liver are the reverse of those existing between the human and the canine livers. There are few reports of the effects of constriction of the cava above the liver in the rat. Volwiler, Grindlay and Bollman (1950), without detailing the evidence, made the statement that rats with hepatic congestion caused by caval cellophane ligatures placed just above the liver did not develop ascites. Nayak, Mehrotra and Mangalik (1956) constricted the cava between the liver and the diaphragm with survival in 21 rats; ascites was present in quantities

up to 22 ml. in 13 of these animals. It was greatest at 2–7 days after the operation, which was performed by an abdominal approach, and it tended to disappear by 28 days. In the experiments reported here, a transthoracic approach has been used and the cava constricted between the diaphragm and the heart. Possible complication of the findings by operative trauma in the peritoneal cavity has thus been eliminated.

METHODS

Partial ligation of the cava was carried out with satisfactory survival in 73 rats. The immediate operative mortality was above 50 per cent, and animals dying during the operations have not been included in this total. A satisfactory degree of constriction was achieved in 55 rats. On most operating days, 2 additional rats were subjected to thoracotomy and tracheotomy alone, to act as controls in the subsequent weighings. This accounted for another 19 rats which need not be further identified. Male and female albino Wistar rats of about 200–250 g. were used, and the operations were done under conditions of cleanliness but not of sterility. Intraperitoneal injections of 1000 units of benzyl penicillin were given on the day of operation and for 5 days thereafter. The animals were weighed before operation and frequently afterwards. Laparotomies were carried out on 8 rats between the 12th and 20th post-operative days to detect possible ascites. In the first week 12 rats died and 2 others died of bronchiectasis many weeks after operation; these have been included in the series. The rest were killed at various times from 5 days to 46 weeks after operation; full histological examinations were carried out. In 2 instances neoprene-latex casts of the hepatic-venous tree were made (Fig. 12).

Right thoracotomy was performed under ether anaesthesia, sometimes supplemented by basal anaesthesia with about 5 mg. of nembutal, through an L-shaped incision through the 6th and 7th costal cartilages and the 7th intercostal space (Fig. 1). Before the chest was opened a tracheotomy was established between the sternomastoid muscles about the level of the lower border of the thyroid. An oxygen-ether mixture was supplied at a rate of about 100 ml./min. through a No. 1 polythene tube as an endotracheal catheter which was passed down to the carina. By this method a rat can be maintained for an indefinite period with the chest open, and respiratory excursions are reduced. By the light of a head lamp the cava was identified. The portion of the inferior vena cava between the diaphragm and the heart is about 3 cm. long, mobile in the mediastinum and accompanied by the right phrenic nerve. In applying the constrictions the mediastinum was perforated. Two forms of constriction were used to close the cava partially. Originally Mackenzie's neurosurgical tantalum clips were applied partly closed, with or without previous application of talc or cellophane to obtain gradual narrowing by scarring. In the last 42 animals of the series, a ligature of thin commercial-cellophane film about 0·5 cm. broad was inserted with a suitably curved aneurysm needle (Fig. 1). This ligature was tied as tightly as the strength of the cellophane would permit round a smooth wire of 15 gauge (1·9 mm. diameter) which was then removed. Before closing the chest the endotracheal catheter was removed and a No. 3 polythene tube (1·5 mm. bore) with a bevelled end was substituted in the tracheotomy. The other end of the tube was attached to a 20 ml. syringe, partly filled with air or oxygen. Artificial respiration was given by an assistant working the syringe while the chest was closed, care being taken not to inflate the lungs too much and to secure closure in full expansion. It was found impossible to reinflate the lungs adequately through a tube of smaller bore which might have been passed through the larynx. Tracheotomy was thus an unavoidable part of the operation although the presence of the incision prolonged the stage of recovery. The tracheotomy wound was closed loosely; tight closure caused respiratory embarrassment and wounds left open became septic.

RESULTS

The main complications of the operation were shock and respiratory embarrassment. Serious sepsis rarely occurred at the sites of incision, which eventually healed well (Fig. 2). After an initial fall, the weights of the animals returned to

pre-operative levels in 3 weeks and the rats remained healthy thereafter, their weights being similar to those of the controls.

It was not possible to correlate any part of the weight graphs of the animals with the degree of closure of the thoracic cava, the degree of ascites or other features found at post-mortem. A sex difference in reaction was not observed.

Satisfactory constrictions were obtained both by clips and by ligatures in 55 rats. In the remaining animals, in which there were no significant hepatic lesions or ascites, the extent to which the cava had been narrowed was less than

FIG. 1.—Transthoracic ligation of the inferior vena cava with tracheotomy. Oxygen is supplied through an endotracheal tube while the chest is open. An aneurysm needle has been passed through the mediastinum and round the inferior vena cava above the diaphragm. The right phrenic nerve is seen on the cava.

half the normal diameter of 2–3 mm. Animals did not usually survive if the cava was narrowed by more than three-quarters at operation and the satisfactory results were obtained with intermediate degrees of constriction. The use of cellophane and talc increased the degree of constriction postoperatively only slightly (Fig. 3) but in many cases thrombosis at the site of ligation or in the intrahepatic portion of the cava (Fig. 4) further reduced the lumen above the liver or restricted retrograde flow to collaterals. There was no evidence during the life of the animals or in the organs examined post-mortem that the kidneys or the hind quarters were damaged as a result of the venous narrowing. Venous thrombosis did not occur at sites distant from the ligatures. Collateral veins were found enlarged after 2–3 weeks, superficial epigastric, internal mammary,

azygos and ascending lumbar veins being the most important channels. The degree to which individual collateral routes were developed varied considerably from one animal to another. The only tributaries of the vena cava between the liver and the heart are diaphragmatic veins and these were often grossly enlarged. In one instance in which the cava was blocked by thrombi both at the site of ligation and within the liver, a diaphragmatic vein provided the whole venous drainage of the liver. Severe hepatic lesions were found, however, in animals in which the cava was not completely closed at the level of the ligature and was fully patent elsewhere.

The liver lesions

The animals have been arranged in 4 groups according to the times of death.

Group I—16 *rats*, 12 *died and* 4 *killed at varying times up to* 1 *week after operation.*—Immediately after operation the liver became moderately congested weighing about twice the normal of 3–4 per cent of total body weight. The liver cells of centrilobular areas appeared shrunken with watery vacuolation of the cytoplasm within a few hours, and after 48 hr. the cells had undergone hyaline necrosis in these sites (Fig. 5), round the junctions of intercalated veins (Fig. 6) and in other non-portal areas. Although the outlines of the cell cords could be distinguished for a few days in the necrotic areas, the affected zones were sharply defined from the surviving liver cells. At the end of 120 hr., resorption of the

EXPLANATION OF PLATES

FIG. 2.—Rat killed 42 days after tracheotomy and ligation of the cava with cellophane. The lungs have been removed to show the cellophane band. The tracheotomy has healed, but the tracheal rings have receded from the site of the incision. The liver is dark and swollen and the spleen is enlarged.

FIG. 3.—Transverse section of cellophane ligature which has narrowed the cava to about half its normal diameter. Eighteen weeks after operation. H. & E. ×13.

FIG. 4.—Organised spontaneous thrombus in hepatic cava in rat killed at 31 days. H. & E. ×11.

FIG. 5.—Centrilobular necrosis 48 hr. after ligation of the cava. The necrotic area is round a central venule (above right) and is well defined from the rest of the lobule in which the cells show " watery " vacuolation. H. & E. ×115.

FIG. 6.—Non-portal necrosis in liver of rat dying 60 hr. after caval ligation. The area chiefly affected is at the junction of two intercalated veins and extends down to a central venule. The involvement of the corresponding lobule is asymmetrical. H. & E. ×80.

FIG. 7.—A subcapsular zone of haemorrhagic necrosis extending out from a small intercalated vein in a rat killed at 42 days. H & E ×80.

FIG 8 —An area similar to that shown in Fig 7 from a rat killed at 31 days. In this reticulin preparation, obliteration of a central vein can be seen. Footes stain ×95.

FIG. 9.—Dilated lymphatics in the hilum of a lymph node from the porta hepatis of a rat killed at 42 days. The sinuses of the node are also enlarged. H. & E. ×13.

FIG. 10.—Peritoneal fibrous tag on the surface of the liver of rat killed at 18 days in which ascites was greatest. H. & E. ×270.

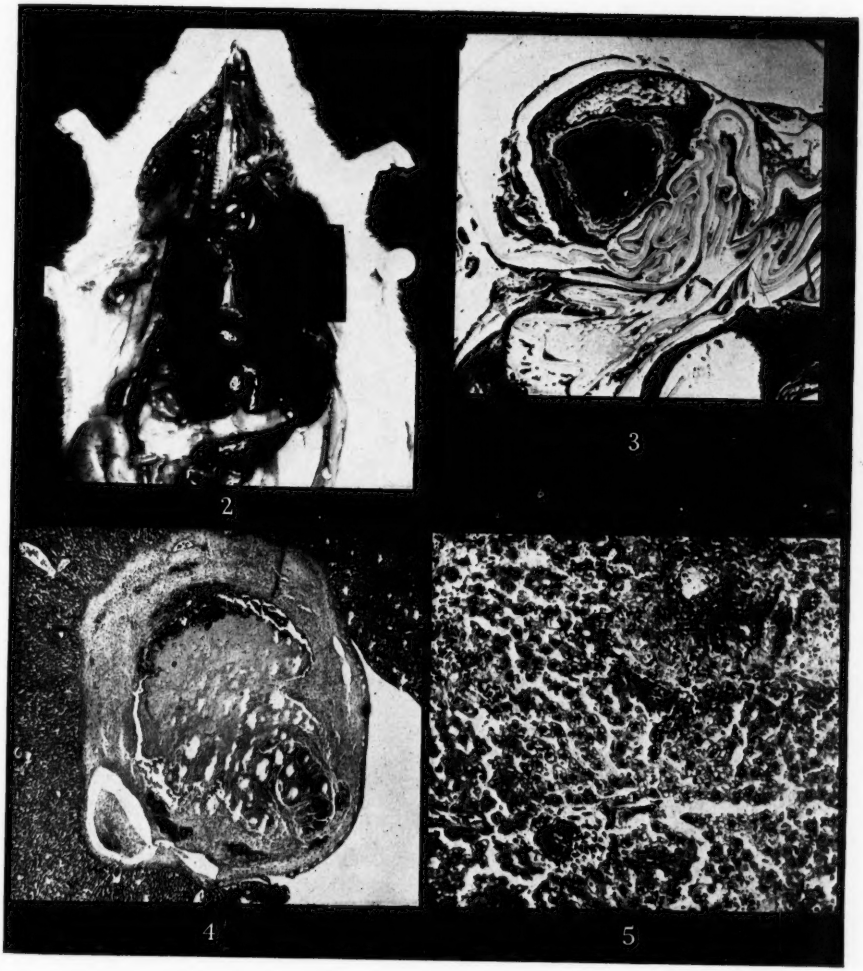
FIG. 11.—Fibrous scar at the junction of intercalated and hepatic veins. It contains macrophages and a few small cysts ; 18 weeks after operation. H. & E. ×80.

FIG. 13.—Areas of persistent haemorrhagic necrosis along the surface of the left lateral lobe (above) adjacent to the median lobe. Groups of dilated sinusoids and dilated central venules can be seen. Rat killed at 18 weeks. H. & E. ×16.

FIG. 14.—A clump of biliary cysts (cholangio-fibrosis) in the liver of a rat killed at 18 weeks. H. & E. ×170.

FIG. 15.—Large cystic spaces, probably vascular, developed in the slow organisation of a superficial haemorrhagic area at 19 weeks. H. & E. ×85.

FIG. 16.—Dilated lymphatics round an hepatic artery in a large portal tract in a rat killed at 18 weeks. H. & E. ×90.



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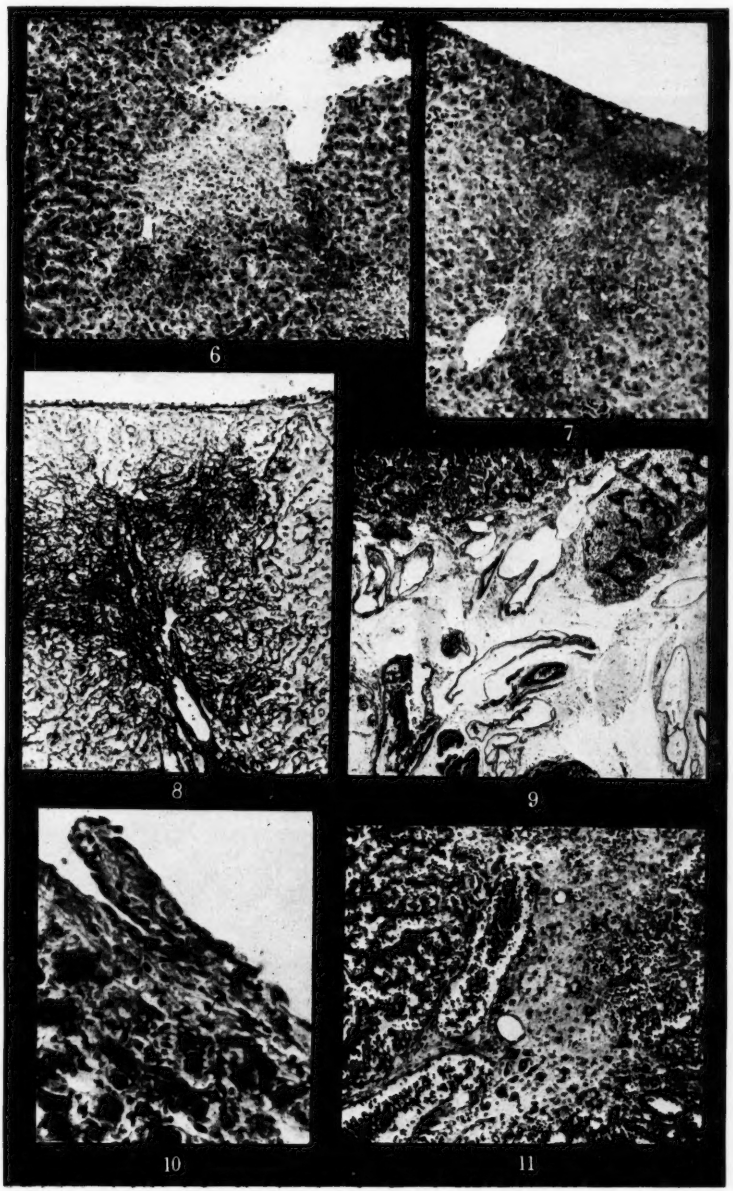

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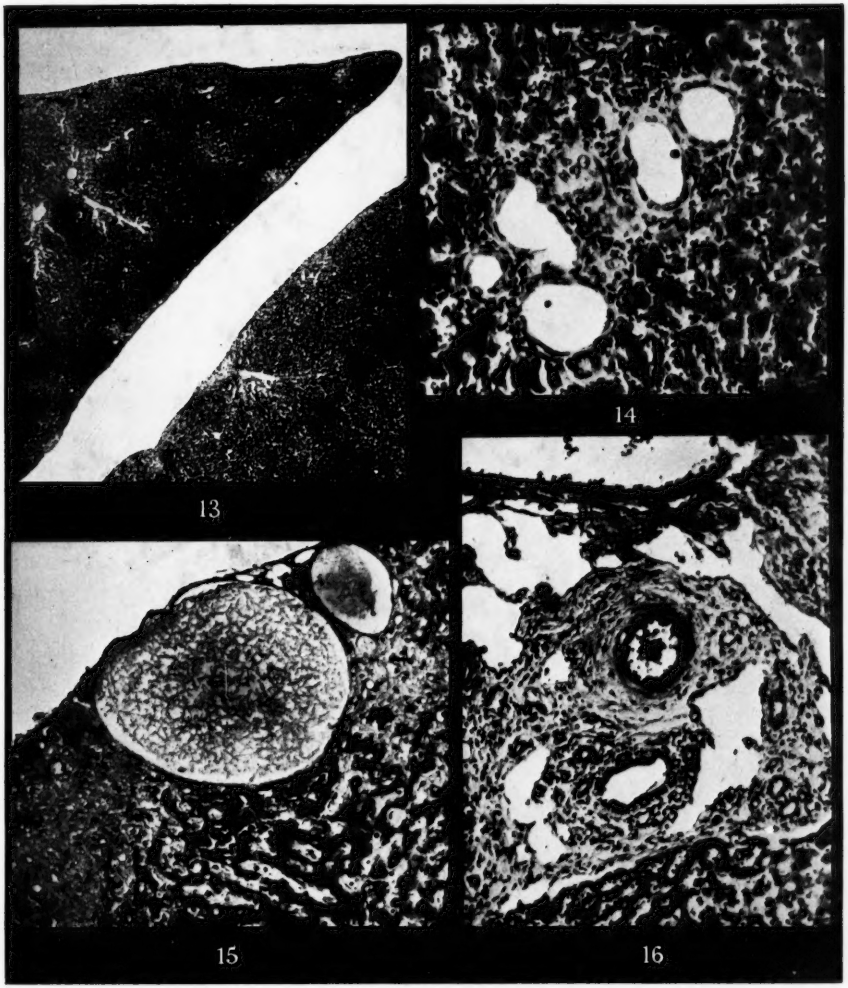

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dead liver cells had begun and Kupffer cells and endothelial cells of the central venules were often swollen. Oedema was sometimes marked round central venules and the outlines of their walls were blurred. The livers were at first severely oedematous and the tissue spaces of the portal tracts and of the hepatoduodenal liagment were distended with fluid. Spaces of Disse were not seen. After a few days, as the initial congestion became less and the interstitial oedema subsided, portal-tract and hilar lymphatics became enlarged and the hilar lymph nodes were swollen and red. Ascites was not observed during the first week.

Group II—11 *rats killed at varying times after* 1 *week and up to* 6 *weeks after operation.*—The livers were increased in weight by 50 per cent of normal; their surfaces were granular with patches of subcapsular haemorrhage and they lacked the normal peritoneal sheen (Fig. 2). The hilar lymph nodes and lymphatics were frequently enlarged; about 3 ml. of clear or slightly blood-stained ascites was present in 5 of the animals. There were thrombi in the hepatic cava in 2 cases (Fig. 4). Microscopically the liver changes were characterised by repair of the process described in the previous group. At 10 days, necrotic material had been largely but not completely removed from the affected areas, which now consisted of blood lagoons with occasional surviving liver cells; a little fine reticulin sometimes preserved the outlines of the liver cells. At all stages in the period, small foci of necrosis were seen which seemed to have been newly produced and not to have persisted from the initial period. The foci were sometimes related to central venules occluded by proliferated fibrous tissue (Fig. 8). The walls of the hepatic veins were increased in thickness by deposition of collagen up to 3 times their normal thickness. In the larger hepatic veins, mural thrombi were occasionally found. Collagen had also begun to form in the liver tissue adjacent to the hepatic veins and in one of these scars a few irregular microcysts of biliary epithelium (cholangio-fibrosis) were found (Fig. 14). Throughout the period, repair of damage to the subcapsular regions was retarded in comparison with the rest. The subcapsular zones consisted of areas of interstitial haemorrhage containing a little irregular reticulin and a few surviving liver cells (Fig. 7 and 8). The central venules draining such areas were sometimes found occluded by fibrous tissue. Lymphatics were large and numerous in the portal tracts and were also increased round the hepatic veins and at the porta (Fig. 9). Lymphatics were not found in the external capsules of the livers. In one animal only, in which there was a serosanguineous effusion of 4 ml. in the peritoneal cavity, peritoneal tags were present (Fig. 10). They were scanty.

Group III—15 *rats killed at varying times after* 6 *weeks and up to* 14 *weeks after operation.*—The livers were a little smaller than those of the previous group and showed variable degrees of congestion and superficial haemorrhage. The surfaces were granular. Lymphatic dilatation was less than in the previous group. There were thrombi in the hepatic cava in 5 cases. The hepatic veins could be recognised as thick by the naked eye. Microscopically small fibrous plaques extended out from the sclerosed hepatic veins into the liver parenchyma.

Fibrous plaques were also found round intercalated veins particularly at their junctions (Fig. 11). In a few instances there was sclerosis of centrilobular areas. Occasionally small septa were formed by the linking up of fibrous areas and in these livers early nodular regeneration could be recognised. The early changes of cirrhosis, which were thus recognisable infrequently, were irregular and did not closely resemble the pattern of pseudolobulation seen in other species.

Neoprene-latex casts of the hepatic veins showed that the architecture of the liver was not greatly disturbed, but the vessels were irregular and dilated and more stumpy than normal (Fig. 12). The irregularities were most marked in the peripheral portions. The left lateral and median lobes were generally worse affected than the right lobes (Fig. 13). In several livers, foci of cholangiofibrosis were found. Fibrosis of the subcapsular areas had begun, but the process was less advanced there than elsewhere. Particularly large areas of haemorrhagic necrosis were found under the capsule of the left lateral lobe in one liver in which the corresponding vein was blocked by an organised thrombus. Small foci of recent necrosis were present in most instances. In general, hepatic-parenchymal-cell activity was increased. In the 4 instances in which the cava had been

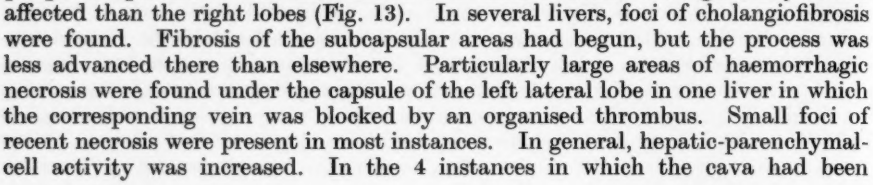

FIG. 12.—Camera-lucida drawing of neoprene-latex casts of hepatic veins of (*a*) normal rat and (*b*) rat killed 11 weeks after partial caval obstruction. The finest vessels shown are sinusoids and only a few of them have been drawn. They enter hepatic veins and intercalated veins as well as cental venules. There is only a little distortion of structure in (*b*) but the vessels are more irregular, dilated and stumpy than in (*a*). ×15.

narrowed least, the fibrosis was less marked than in the other livers, but central venules and the central parts of sinusoids round them were irregularly dilated (Fig. 13).

Group IV—13 *rats*, 11 *killed and* 2 *died at varying times after* 14 *and up to* 46 *weeks after operation.*—The livers weighed about 1·25 times normal and showed gross and histological characters similar to those in group III but more uniform and more firmly established. In several livers, cirrhotic features were present but they were not advanced in degree. Foci of cholangiofibrosis were often seen (Fig. 14). In the scarred areas (Fig. 11) there were macrophages in varying numbers containing lipofuscin pigment of ceroid type. Iron pigment was also present in macrophages in portal tracts and elsewhere—a finding which was generally absent in the earlier groups. In several instances there were foci of haemopoiesis in portal tracts. Foci of necrosis persisted, especially in peripheral areas (Fig. 13). These zones were organised in other instances, the loose fibrous tissue containing wide sinusoidal channels; in some cases cystic spaces (Fig. 15) were also present which were probably vascular in character. The lymphatics

were usually less dilated than in the earlier stages but remained considerably widened (Fig. 16). Thrombi were present in the hepatic cava in 7 cases.

Ascites

Ascites was found only between the 2nd and 6th post-operative weeks (*i.e.* in group II) and was always small in amount. The first observation of ascites was in an animal killed at 12 days, in which resorption and organisation of necrotic foci had begun; the hilar lymphatics and lymph nodes were enlarged. The ascites amounted to about 3 ml. The findings were similar in 4 other animals killed between that time and the end of the 6th week. A few fibrous peritoneal tags were present on the surface of the liver of one of these animals in which the greatest amount of ascites was found (Fig. 10). The ascites was greatest in animals killed 2–3 weeks after operation. In the 6 other rats killed in this group, ascites was absent or minute, but the hilar lymph nodes were enlarged in all (Fig. 9). To extend the range of these observations, laparotomies were carried out on 8 other rats which were allowed to recover. Although only a little fluid was encountered in the peritoneal cavities when they were opened, there was a copious transudation of clear fluid amounting to a few ml. from all surfaces of the congested livers when they were handled at laparotomy. Pleural effusions were not encountered in any animal killed more than a few days after operation.

Effects on the portal-venous bed

The rats were killed by an overdose of anaesthetic and the portal vein was often inspected before death. It was dilated. No attempt was made to define portal-systemic anastomoses. The spleen was enlarged in nearly all cases; 27 of the spleens were weighed and 43 were examined histologically. At death during the first few days after operation, the spleens weighed up to twice the normal which is approximately 0·3 per cent of total body weight. The increase was mainly due to congestion. Congestion diminished later in the week and the normal foci of haemopoiesis with megakaryoctyes increased at the same time. In the second group (*i.e.* 1–6 weeks) the weights ranged up to 3 times normal and the largest spleens were found in animals with severely damaged livers. Both Malpighian bodies and red pulp were increased in amount in this group. The increase in the red pulp was characterised by an increase in cells with a basophilic and pyroninophilic cytoplasm, some of which were probably "pulp cells" and others haemopoietic. Similar cells were found in the intima of the splenic veins. Brown pigments both positive and negative to Perl's reaction were often present in increased amounts. The histological findings in animals surviving longer (groups III and IV) were similar but the spleens tended to be smaller. Variable degrees of congestion were found and this influenced the weights. The splenic structure had undergone a little fibrosis in the animals surviving longest but this was considerably less than that commonly present in the fibroadenie of human portal hypertension. It was not possible to relate the spleen weights accurately to the degrees of liver damage encountered in the animals.

DISCUSSION

In the experiments of Nayak *et al.* (1956) ascites was not invariably present but it was earlier in appearance and greater in amount than in the present series. These authors narrowed the cava below the diaphragm to one-third of its normal

diameter, which is 4 mm., at this level, *i.e.* to a similar degree to that reported above. Thus the discrepancy between the results in the 2 series is not due to a difference in the degrees of constriction originally imparted. Fibrosis round the cellophane ligature and subsequent thrombosis, which enhanced the degree of obstruction as described above, caused no apparent regular increase in the severity of the ascites. It seems more likely that handling of the liver and peritoneum during the abdominal operations of Nayak and his colleagues promoted the prompter and more copious formation of ascites in their series. The transthoracic approach used in the present series eliminated this complication and the results probably represent better the effects of venous congestion alone. The use of tracheotomy increased the operative and post-operative hazards, but with the administration of penicillin it was possible to obtain a satisfactory number of survivals.

The amounts of ascites in the experimental animals were small, below 2 per cent of total body weight. They are approximately equal to those reported in rabbits and cats and much smaller than those in dogs (up to 35 per cent). Ascites was found only between the 2nd and 6th post-operative weeks and not in all animals killed in this period. Ascites formed copiously however, on handling the livers at the laparotomies performed at this time and an underlying tendency to its formation was demonstrated. This observation is significant in two connections. In the first place it may explain the difference between the results published here and those of Nayak and his colleagues. Secondly it emphasises the role of transudation of lymph or tissue fluid from the surface of the liver in the formation of ascites in hepatic venous congestion. Bolton and Barnard (1928) reported endothelial buttons on the peritoneal surfaces of the livers of cats in which the inferior vena cava had been obstructed in the thorax and they associated these buttons with the ascites they found. In the present experiments a few similar subperitoneal tags were found on the liver of the rat in which the greatest spontaneous ascites was recognised (Fig. 10). Similar tags can be found in human hepatic venous congestion (unpublished observations) and possibly they give an indication of the amount of fluid produced at this site. The occurrence or absence of peritoneal tags may perhaps offer a useful indication of the part played by transudation in the formation of ascites in liver disorders of other types. In chronic venous congestion in man, the lymphatics of the hepatoduodenal ligament are dilated (Baggenstoss and Cain, 1957). In the present experiments the corresponding lymphatics were dilated (Fig. 9) but this seemed to be of less importance than the congested liver in causing the effusions. Mallet-Guy, Devic, Feroldi and Desjacques (1954) showed that transudation from the liver surface is the main cause of the massive ascites that occurs in experimental hepatic venous congestion in the dog. These authors transposed parts of the liver into the thorax after rendering them congested by narrowing of the appropriate veins; a pleural and not a peritoneal effusion resulted. The massive development of ascites in the dog may be associated with the large extent of the capsular lymphatics in the liver of that animal. This plexus is virtually absent in the rat and the ascites of hepatic venous congestion is correspondingly slight. In man the state of affairs is probably intermediate. In all 3 species the production of ascites in venous congestion of the liver, whether in large or small amounts, seems to be brought about in large part at least by transudation of tissue fluid or lymph from the surface of the liver.

The hepatic veins of man contain moderate amounts of muscle ; those of the dog are heavily muscularised and surrounded by an exceptionally rich lymphatic plexus. As a consequence of this structural character, it is likely that under physiological conditions the lymphatic outflow from the liver reaches exceptionally high levels from time to time in the dog. This element does not enter into the pathogenesis of ascites in the rat in which the hepatic veins have thin walls with lymphatics on a similar scale to those in man. Considerations of this sort may account for species variations in reaction to caval obstruction, as for instance in the duration of the experimental ascites. The ascites remits spontaneously within a few weeks, in rats, rabbits and cats but not till after many months in dogs. Because of the large amounts of fluid formed, the production of experimental venous congestion of the liver of dogs is of particular value in the study of therapeutic measures which might modify ascites, but as regards the pathogenesis the results may be misleading if applied to man without taking into account species variations such as those encountered in the present experiments.

Since ascites was not present in animals killed after the 6th post-operative week, a balance in the total circulatory arrangements seems to have been struck by that time. A comparable balance was not struck in the case of the liver parenchyma. The experimental procedure caused a liver lesion which progressed slowly by repeated necroses and later by formation of fibrous tissue. In some cases nodular regeneration had begun and the condition could justifiably be termed a cirrhosis. Severe cirrhosis was not produced ; hepatic-venous casts showed little distortion of the normal vascular pattern, although the vessels were irregular, wider and more stumpy than normal (Fig. 12). There was a considerable and progressive fibrous thickening of the walls of the hepatic veins and new fibrous tissue extended into the adjacent liver tissue.

Elias and Popper (1955) have described differences in the drainage of sinusoids between the human and the rat livers which have a bearing on the location of the necroses and on the character of the subsequent cirrhosis. In man the sinusoids terminate exclusively in central venules and there is a bottleneck in the venous drainage at this level, which is evidenced by the notably symmetrical and regular pattern of chronic venous congestion. In the rat on the other hand, many sinusoids terminate in intercalated veins and some even in hepatic veins (Fig. 12), so that centrilobular bottlenecks do not occur. The foci of necrosis, which were found at all stages of the experimental series but especially in the initial phases, were regularly non-portal in distribution (Fig. 5) but they were not exclusively centri-lobular and were often asymmetrical in their distribution round central venules (Fig. 6). Areas round the junctions of intercalated veins seemed to be selectively affected (Fig. 6) and it is possible that relative bottle necks in the hepatic venous drainage of the rat are found at that level.

Chronic venous congestion of the liver in man is characterised by centrilobular sclerosis and the most important way in which cardiac cirrhosis is brought about is by the linking of these areas to one another or to portal tracts to form the septa of pseudolobulation. In the rat livers in which centrilobular sclerosis was uncommon, because of the better venous drainage of this area, the pattern of pseudolobulation was not found. Where septa were present, they generally linked fibrous plaques round intercalated veins (Fig. 11). While the formation of the septa of pseudolobulation is the common way in which human cardiac cirrhosis is produced, one sometimes finds that individual lobules have been

destroyed because their central venules have been obliterated by a proliferation of fibrous tissue within the lumen. In the rats, the cirrhotic changes were most often brought about in this second way. Occlusion of central venules was encountered most frequently in the subcapsular zones (Fig. 7 and 8) and since some of the lobes of the rat liver are thin, this sometimes included a considerable fraction of the liver substance. The necrosis in these regions was haemorrhagic (Fig. 7 and 13), and organisation was delayed there in comparison with other sites. Nayak *et al.* (1956) describe similar areas as infarcts and this is in keeping with the part played by vascular occlusions in their formation. Kawano (1957) has reported a tendency to more severe involvement of the superficial areas of the liver in similar experiments on rabbits. Under the experimental conditions of circulatory embarrassment, blood may have been deviated from these superficial areas through central liver tissue via the short-circuit route of Daniel and Pritchard (1951) in these multilobed animal livers. This cannot be the whole explanation however, for a similar phenomenon occurs in the unitary liver of man in which the anatomical basis for such shunts does not exist. Certain characters of the vascularisation of the peripheral parts of the liver are probably important in this respect. In casts of the hepatic veins of 2 animals of Group III, the greatest distortion of venous pattern was found in the peripheral zones. Subcapsular lobules are, of course, devoid of sinusoidal anastomoses on their outer aspects and some of the smaller veins of the region are unduly long in the rat (Wakim and Mann, 1942). The thin capsule of the liver is practically devoid of vessels and new vessels developed slowly in the haemorrhagic zones. Eventually, when organisation was advanced, large sinusoidal vessels were found and some cystic spaces encountered (Fig. 15) were probably of this nature also.

The liver damage was occasionally enhanced by the formation of thrombi in hepatic veins and foci of necrotic liver tissue may have been partly responsible for the formation of the thrombi which were found in the hepatic cavas of many of the rats (Fig. 4). The cava of the rat is practically an hepatic vein at that level and its wall is so thin that devitalisation of underlying parenchyma might be expected to have an effect on the endothelium. We may presume that the operative stenosis of the cava above the liver also promoted thrombosis in the hepatic portion by interfering with the free flow of blood through the vessel. With these exceptions the experimental procedure did not lead to thrombosis in the experimental animals, and there was no evidence that the occlusions of the central venules within the liver were thrombotic in origin. They appeared to be simply one element of a phlebosclerosis which was found throughout the hepatic-venous system.

The occurrence of foci of cholangiofibrosis in many of the livers in the later groups (Fig. 14) following the purely mechanical procedure used in these experiments is an indication of the non-specific aetiology of this lesion. Cholangiofibrosis has a premalignant character (Gupta, 1956), but this potentiality was not realised in any of the animals in this series.

Portal circulation

The spleen was regularly enlarged to an extent comparable with that produced by Reynell (1952) by subtotal constriction of the portal vein in the rat. The enlargement was due mostly to an increase in haemopoiesis and in pulp cells.

Cameron and de Saram (1939) considered that splenic-pulp hyperplasia was independent of portal hypertension in the rat and Kent, Popper, Dubin and Bruce (1957) have sought to link its occurrence in ethionine-induced cirrhosis with an elevation in γ-globulin. Since it is difficult to distinguish pulp cells from haemopoietic cells and since megakaryocytes and other readily identified haemopoietic elements were greatly increased in the present material, it seems likely that much of the increase in the weights of the spleens was due to an exaggeration of the normal haemopoiesis of this organ in the rat. This may have been brought about by an element of stagnation in the portal circulation. Fibrosis is more generally acceptable as an indication of portal hypertension; fibrosis was less marked in the spleens of this series than in those described by Kent and his colleagues in which the liver damage was more rapidly progressive. Reynell (1952) has shown that experimental portal hypertension in the rat tends to diminish spontaneously and the spleens of the animals killed in the later stages of the experiments tended to be smaller than those of animals of the earlier groups.

The portal vein was regularly dilated in the experimental animals and the effects of the post-sinusoidal block were clearly transmitted to the portal system. Uncomplicated human portal-venous obstruction is usually not accompanied by ascites (Gibson and Richards, 1955) but transudation may occur from the portal-venous territory, when cirrhosis is complicated by other factors. There was no evidence that the ascites in the present experiments was produced from any site other than the liver, where the increment in venous pressure was probably greatest.

SUMMARY

A technique is described for obstructing the inferior vena cava above the liver in the rat using a transthoracic approach and tracheotomy. A satisfactory degree of constriction, *i.e.* to less than half the normal diameter, was obtained with survival in 55 rats by the use of neurosurgical tantalum clips or cellophane ligatures. Animals were killed at intervals up to 46 weeks after operation.

A collateral venous circulation developed which sufficed for the kidneys and hind parts of the animals. The livers were severely congested. Ascites of less than 2 per cent of body weight was found in several but not in all animals killed at times from one up to 6 weeks after operation. There was marked dilatation of the lymphatics of the liver in all the rats. At laparotomies performed on 8 other animals at similar times after operation, there was a copious transudation of fluid from the surface of the liver on handling the organ. When ascites develops in chronic venous congestion of the liver in the rat it apparently arises chiefly in transudation from the surface of the liver and this is probably the case in other species too.

Splenic enlargement was regularly present and was due to increased haemopoiesis, pulp hyperplasia and congestion.

A slowly progressive liver lesion with repeated episodes of focal necrosis and culminating in a mild form of cirrhosis with early nodules of regeneration was produced by the caval obstruction. There was phlebosclerosis of the hepatic veins and some central venules were obliterated by fibrous tissue with consequent destruction of lobules. This process was most marked in the subcapsular zones.

Plaques of fibrous tissue also formed at sites of previous necroses, particularly at the junctions of intercalated veins, and septa sometimes formed by the coalescence of these plaques. The findings are discussed in relation to those reported by others in similar experiments on other species particularly dogs, and some comparisons are drawn with human diseases.

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THE EFFECT OF BACTERIAL ENDOTOXIN ON RESISTANCE OF MICE TO ECTROMELIA

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The complex pharmacological and pathological actions of endotoxins from Gram negative bacteria have been studied intensively in the last few years. Among other important facts brought to light it appears that the susceptibility of animals to the lethal effects of Gram negative bacteria is increased for a few hours after the inoculation of endotoxin. This increased susceptibility is superseded by a phase of increased resistance which lasts for more than a week (Rowley, 1955; Landy, 1956). Experiments are here described which showed that mice inoculated with endotoxin followed by large doses of ectromelia (10^6LD_{50}) survive longer than control mice which receive only the virus. This result was expected by analogy with that obtained when mice pre-inoculated with endotoxin are challenged with bacteria. In other experiments in which small doses of ectromelia were used, mice pre-treated with endotoxin died sooner than control mice. Essentially the same result was obtained by treatment of mice with saccharated iron oxide, a treatment selected by reason of its effect on the reticulo-endothelial system (R.E.S.).

METHODS

The mice used for most experiments belonged to a strain of white mice (TO strain) bred at the National Institute for Medical Research, Mill Hill, since 1953 and obtained originally from Dr. Herdis von Magnus of the State Serum Institute, Copenhagen. As received these mice were free from the TO and other strains of mouse poliomyelitis and became known as the "TO free" strain of mice. They have since acquired mouse poliomyelitis and are now known as "TO" strain of mice. In a few experiments, mice of the VS strain were used. This strain originated from Webster's (1937) bacteria-susceptible virus-susceptible sub-line of a colony of mice bred as a closed colony at the Institute since 1949 and during this time no selective breeding was practised to maintain their high susceptibility to infection.

Two types of endotoxin from Gram negative bacteria have been used. One was dried semi-purified endotoxin from *Chromobacterium prodigiosum*, kindly supplied by Dr. J. H. Humphrey. Since it had originally been prepared as a standard for pyrogen, we refer to it as pyrogen. In the experiments to be described, it was injected intraperitoneally in a dose of 50 μg. contained in 0·2 ml. water. The other endotoxin was a Seitz filtrate of a formolized growth of *Salmonella typhimurium*, strain S4 (Gledhill and Niven, 1957). This endotoxin, termed S4 filtrate, was injected in a dose of 0·2 ml., intraperitoneally in most experiments. Saccharated iron oxide (Ferrivenin of Benger Laboratories) was injected intravenously in a dose of 0·3 mg. contained in 0·2 ml. of saline on 4 consecutive days.

The strain of ectromelia used had been isolated from a spontaneous outbreak in 1951 and after a few mouse passages was stored at $-76°$ as a 10 per cent liver spleen suspension in 50 per cent serum broth-saline. This was used as seed for the present experiments for which 2 batches of liver/spleen suspension were prepared. These batches were stored in 2 ml. amounts in screw-capped bottles at $-76°$ and drawn as required for experiments.

RESULTS

Groups of 20 weanling mice (strain TO) were treated in the following ways: Mice of one group received an inoculation of 50 μg. pyrogen intraperitoneally and another group received an inoculation of 0·2 ml. S4 filtrate intraperitoneally. A third group was inoculated intravenously with Ferrivenin on four consecutive days. After 24 hr. mice of these groups and a group of untreated control mice were inoculated intraperitoneally with about 10^6LD_{50} ectromelia virus. The results are shown in Table I from which it is clear that all groups of pre-treated

TABLE I.—*Increased Survival Time of Mice Pre-treated with Endotoxins and Saccharated Iron Oxide (Ferrivenin) and Infected with* 10^6LD_{50} *Ectromelia*

Mice pre-treated with	Proportion of survivors $1\frac{1}{2}$ days after virus inoculation	Probability that mice of treated groups have same resistance as controls		Average survival time (days)
		χ^2	p	
Pyrogen	20/20	12·5	<0·001	2·53±0·44
S4 filtrate	20/20	12·5	<0·001	2·29±0·35
Ferrivenin	19/20	9·6	<0·01	2·20±0·34
Nil (controls)	9/20	—	—	1·75±0·37

Pyrogen 50 μg. i.p. 24 hr. before virus.
S4 filtrate 0·2 ml. i.p. 24 hr. before virus.
Ferrivenin (saccharated iron oxide) 0·3 mg. in 0·2 ml. i.v. on 4 consecutive days, last dose 24 hr. before virus.
Virus: 10^6 LD_{50} i.p.

mice survived longer than the controls and the differences are small yet highly significant. This result accords with the hypothesis that phagocytosis of virus by the R.E.S. is stimulated by these agents.

In another experiment using similar groups of mice, mice of two groups were inoculated intraperitoneally with 0·2 ml. S4 filtrate and 23 hr. later mice of two other groups were similarly inoculated. After 1 hr. one of the groups inoculated 24 hr. previously and one of the groups inoculated 1 hr. previously were inoculated with 10^6LD_{50} virus and the remaining two groups with 10^3LD_{50} virus. One group of control mice received the higher dose of virus and another received the lower dose. The results of this experiment are shown in Table II. As in the previous

TABLE II.—*Contrary Effect of Endotoxin upon Survival Time of Mice Infected with* 10^6 *and* 10^3LD_{50} *virus*

Dose of virus i.p. (LD_{50})	Time interval between endotoxin and virus	Survivors $2\frac{1}{2}$ days after virus inoculation	Survivors 6 days after virus inoculation	Probability that mice of treated groups have same resistance as controls		Average survival time (days)
				χ^2	p	
10^6	24 hr.	17/20	0/20	8·4	<0·01	3·33±0·55
	1 hr.	13/20	0/20	2·5	<0·2	2·81±0·54
	Control	7/20	0/20	—	—	2·68±0·38
10^3	24 hr.	20/20	7/20	5·0	<0·05	5·88±1·18
	1 hr.	20/20	3/20	12·2	<0·001	5·38±0·84
	Control	20/20	15/20	—	—	7·03±1·48

Endotoxin (S4 filtrate) 0·2 ml. i.p.

experiment, the pre-treated mice which received $10^6 LD_{50}$ of virus survived longer than the corresponding controls, even when the inoculation of the endotoxin preceded that of virus by only one hour. Rather surprisingly, the groups of pre-treated mice which received the smaller dose of ectromelia died sooner than the corresponding controls. In view of this result, groups of 20 mice were inoculated intraperitoneally with 50 μg. pyrogen and 0·2 ml. S4 filtrate and 24 hr. afterwards these mice and mice of a control group were inoculated by the same route with about 1 LD_{50} of ectromelia virus.

In another experiment similar groups of mice were inoculated with 0·2 ml. S4 filtrate and with saccharated iron oxide according to the previous dosage schedule. These groups and a control group were also inoculated with 1 LD_{50} of virus 24 hr. after the inoculation of endotoxin and of the last dose of saccharated iron oxide, The combined results of both these experiments are shown in Table III. It will

TABLE III.—*Decreased Survival Time of Mice Pre-treated with Endotoxins and Saccharated Iron Oxide (Ferrivenin) and Infected with 1 LD_{50} Virus*

Mice treated with	Proportion of survivors 10 days after virus inoculation	Probability that mice of treated groups have same resistance as controls	
		χ^2	p
Pyrogen	0/20	22·5	<0·001
S4 filtrate	3/20; 5/20	14·0; 9·2	<0·001; <0·01
Ferrivenin	5/20	9·2	<0·01
Controls	13/20; 15/20	—	—

Treatment with Pyrogen, S4 filtrate and Ferrivenin as in Table I.
Virus: 1 LD_{50} i.p.

be seen that there were significantly fewer survivors 10 days after inoculation of virus in the groups of pre-treated mice than in the controls. In these experiments and others carried out in normal mice and mice pre-treated with S4 filtrate and infected with about 1 LD_{50} of virus, the final mortality was invariably higher in the pre-treated mice than in the normal mice. Thus, the mortality in pre-treated mice in all experiments with this dose of virus was 151/159 or 95 per cent compared with 110/200 or 55 per cent for the controls ($\chi^2 = 69·8$). To avoid misconception it should be understood that all mice inoculated with 1 LD_{50} of virus are infected with ectromelia, as judged by the development of immunity in survivors. In fact 1 LD_{50} of virus represents a dose of about 10 ID_{50} to untreated mice. Challenge of surviving normal mice and endotoxin pre-treated mice with $10^4 LD_{50}$ of virus 14 days after the inoculation with 1 ID_{50} does not indicate that pre-treatment with endotoxin has any noticeable influence upon the infectivity of ectromelia virus.

An experiment was carried out to compare the titration of virus in mice inoculated intraperitoneally 24 hr. previously with S_4 filtrate and in normal mice. Weanling mice of strain TO were used as heretofore and also weanling mice of strain VS. The virus was inoculated intraperitoneally (0·2 ml.) in decimal dilutions from 10^{-3} to 10^{-10} into groups containing 10 mice. The result of this experiment is shown in Table IV. It will be seen that in the TO mice, the titration end-point is much more clear-cut in the mice pre-treated with endotoxin and this results in an LD_{50} value of 0·7 log units higher. The end-points of the titrations in both

TABLE IV.—*Titration of Ectromelia Virus in Normal and Endotoxin Pre-treated Mice of Strains TO and VS*

— $\log_{10}$ dilution of virus suspension	Mortality: Mice which died / Mice in group (a) Normal TO	(b) Endotoxin TO	Mean survival time (days) (a) Normal TO	(b) Endotoxin TO	Difference (a)–(b)
10	1/10	0/10	—	—	—
9	3/9	1/10	8·7	—	—
8	4/10	9/10	12·0	9·3	+2·7
7	8/10	10/10	9·5	8·4	+1·1
6	9/10	9/10	7·8	7·8	0·0
5	8/10	10/10	6·9	6·6	+0·3
4	10/10	10/10	6·7	6·3	+0·4
3	10/10	10/10	5·8	5·0	+0·8
Total mortality (log dilutions 3–8)	49/60	58/60			
	(a) Normal VS	(b) Endotoxin VS	(a) Normal VS	(b) Endotoxin VS	
10	0/10	1/10	—	—	—
9	3/10	3/10	8·0	7·7	+0·3
8	10/10	9/9	8·8	7·3	+1·5
7	10/10	10/10	8·0	5·8	+2·2
6	10/10	10/10	7·2	6·1	+1·1
5	9/9	10/10	6·0	5·7	+0·3
4	9/9	9/9	5·2	5·3	—0·1
3	10/10	9/9	4·6	4·6	0·0
Total mortality (log dilutions 3–8)	58/58	57/57			

Endotoxin: 0·2 ml. S4 filtrate inoculated i.p. 24 hr. before virus.
Virus dilutions made in serum saline broth and inoculated 0·2 ml. i.p.

LD_{50}
- Normal TO weanling mice 0·2 ml. $\times 10^{-7.8}$.
- Endotoxin TO weanling mice 0·2 ml. $\times 10^{-8.5}$.
- Normal VS weanling mice 0·2 ml. $\times 10^{-8.7}$.
- Endotoxin VS weanling mice 0·2 ml. $\times 10^{-8.8}$.

classes of VS mice are clear-cut and there is practically no difference in the LD_{50} given by each class (8·7 and 8·8). Thus, if we exclude from the titrations the groups of mice which received dilutions of 10^{-10} and 10^{-9} on the ground that the majority of these mice were not infected, we obtain the following overall mortalities:

Mice	Mortality	
TO normal	49/60 (82 per cent)	$\chi^2 = 5{\cdot}52$ p $< 0{\cdot}02$
TO endotoxin	58/60 (97 ,, ,,)	
VS normal	58/58 (100 ,, ,,)	$\chi^2 = 0{\cdot}48$ p $> 0{\cdot}4$
VS endotoxin	57/57 (100 ,, ,,)	

The last 3 of these mortalities do not differ significantly from one another, whereas all three differ significantly from the first, *i.e.* TO normal. It seems reasonable to conclude that normal VS mice are more susceptible to the lethal action of ectromelia virus than normal TO mice and that whereas the susceptibility of VS mice is very little increased by pre-inoculation of endotoxin, pre-inoculation of endotoxin raises the susceptibility of TO mice almost to that of VS mice.

The time at which endotoxin is inoculated is not a critical factor in the production of increased mortality. For example, a group of 20 mice was inocu-

lated with 0·2 ml. S4 filtrate 48 hr. before about 1 LD_{50} of virus, another group 1 hr. before the virus, a third group 48 hr. after the virus and a control group of 19 mice received only the virus. The mortalities were as follows:

S4 filtrate 48 hr. before virus	19/20	(95 per cent)
,, 1 hr. ,, ,,	20/20	(100 ,, ,,)
,, 48 hr. after ,,	20/20	(100 ,, ,,)
Controls (virus only)	11/19	(58 ,, ,,)

Each of these mortalities is significantly higher than that of the controls. The probability that the first group belongs to the same population as the controls is $p < 0{\cdot}02$ ($\chi^2 = 5{\cdot}57$) and for both the second and third groups the corresponding probability is $p < 0{\cdot}01$ ($\chi^2 = 8{\cdot}11$).

DISCUSSION

The inoculation of mice with endotoxin at first lowers and then raises their resistance to infection with Gram negative bacteria, as we noted earlier. These changes of resistance are generally accompanied by parallel changes in the concentrations of circulating properdin (Pillemer and Ross, 1955; Landy and Pillemer, 1956). Since, however, changes of resistance do not regularly reflect the changes of properdin concentrations in the serum, it is improbable that the concentration of properdin in the blood is the chief factor which governs resistance (Ross, 1958; Howard, Rowley and Wardlaw, 1958).

The power of the R.E.S. to clear the blood of particulate matter is likewise depressed for a few hours and then exalted for more than a week following the intraperitoneal or intravenous inoculation of endotoxin and a variety of particulate substances, such as saccharated iron oxide (Biozzi, Halpern, Benacerraf and Stiffel, 1957). This has suggested that the phagocytic activity of the R.E.S. is one of the principal factors which determine non-specific resistance to bacterial infection. The results obtained with mice infected with large doses of ectromelia (about 10^6LD_{50}) follow the same pattern as in mice infected with bacteria, that is to say, they survive longer if the activity of the R.E.S. has been stimulated, whether by endotoxin or saccharated iron oxide. The results can be explained, as in the case of bacterial challenge, by the fact that the exalted R.E.S. clears the blood of virus more efficiently than the normal R.E.S. and thereby lessens the invasion of vital structures, such as the liver parenchyma. The preliminary period of depression of resistance following the inoculation of endotoxin, which can be demonstrated by bacterial challenge, cannot be clearly demonstrated with ectromelia. This difference is probably related to the fact that the full effect of the bacterial challenge employed by most workers, takes 24 hr. to develop compared with at least 48 hr. with 10^6LD_{50} of ectromelia. Thus, the R.E.S. would be depressed during a smaller proportion of the incubation period with ectromelia than would be the case with the bacterial challenge. It is significant that the sparing effect of endotoxin given 24 hr. before 10^6LD_{50} ectromelia (average survival time 3·33 days) is greater than the sparing effect when it is given only 1 hr. before the virus (average survival time 2·81 days, see Table II).

The explanation of the sparing effect of R.E.S. stimulants would lead one to expect it to be equally manifest with small doses of ectromelia. In fact the

contrary is the case. Mice treated with R.E.S. stimulants are more susceptible to the lethal effect of small doses of ectromelia than normal mice. Virus removed from the blood by the R.E.S. multiplies there. Fenner (1949) observed that " the spleen shows macroscopic changes at least a day earlier than the liver and this corresponds with higher titres of virus found in the spleen each day until death, when the titres of the spleen and liver are approximately the same." Presumably the multiplication may lead finally to such functional impairment that the R.E.S. can no longer contain the virus which thereupon spreads to vital structures. Thus the survival or death of a mouse infected with 1 LD_{50} of virus is chiefly determined during the relatively long incubation period of 7 or more days when the virus impairs the R.E.S. The impairment is opposed by reparative forces, which include enhanced multiplication of R.E. cells and by the rising tide of antibody production.

To account for the anomaly that stimulation of the R.E.S. of mice lowers their resistance to the pathogenic action of ectromelia virus, we assume that the virus multiplies better in the stimulated R.E.S. than in the normal R.E.S. In consequence the functional impairment of the stimulated R.E.S. is an earlier and more frequent result of ectromelia infection than the functional impairment of the normal R.E.S. Howard, Rowley and Wardlaw (1958) make a similar assumption. These authors showed that the stimulation of the R.E.S. by Thorotrast which, as might be expected, increased resistance of mice to *Bacterium coli* infection, decreased their resistance to *Salm. typhimurium*. This species of bacterium differs from *Bact. coli* in its power to parasitize the R.E. cells. They suggested that the increased susceptibility of mice treated with Thorotrast to the pathogenic action of *Salm. typhimurium* is due to an increased capacity of these organisms to multiply in the R.E.S. after stimulation with Thorotrast.

In other virus diseases, especially those in which there is a relatively long incubation period terminated by detectable viraemia at about the time of onset of symptoms, the virus must multiply and produce effects in some tissue during the incubation period to account for its subsequent invasion of the blood-stream and lodgement in vital tissues at the end of the incubation period. It seems improbable that the incubation period is simply the time during which the primary lesion progresses to the point where it secretes sufficient virus to block the R.E.S. and allow a demonstrable viraemia to develop. The primary lesion is usually inconspicuous and yet a very large number of particles is required to produce demonstrable blockade of the R.E.S. Thus, the hypothesis that a virus entering the R.E.S. multiplies in it and damages it might also apply to some other virus diseases. At least it is a hypothesis which takes some account of what might occur during the incubation period. If, in fact, the cells of the R.E.S. are adapted to prolonged low-grade multiplication of many viruses, such viruses would be present over a long period of time at a site close to cells which produce antibody. This might help to explain the persistence of circulating antibody for years after recovery from some virus diseases.

Briody, Hauschka and Mirand (1956) have shown that genetic factors play some part in the resistance of mice to ectromelia. It is interesting that the VS strain of mice, originally bred by Webster for susceptibility to virus diseases (Webster, 1937) proved to be more susceptible to the lethal effect of ectromelia than the TO strain of mice. It is perhaps of greater interest that the susceptibility of the VS strain is not significantly enhanced by pre-treatment with endotoxin

whereas the susceptibility of the TO strain is enhanced by pre-treatment to the point at which it is approximately equal to that of the VS strain. This might suggest that the strain difference in susceptibility, presumably genetical in origin, is located in a difference in the R.E.S. of mice of the two strains. Perhaps the R.E. cells of TO mice only support restricted multiplication of ectromelia virus unless they are stimulated by endotoxin, whereas the R.E. cells of VS mice do not require stimulation to support full multiplication of the virus.

Sugawara (1957) showed that mice on a high protein diet survived a massive dose of ectromelia virus longer than mice on a low protein diet. With smaller doses of virus, the situation was reversed, *i.e.* the mice on a high protein diet died sooner than those on a low protein diet. This reversal of effect resembles our observations and would become essentially the same if a high protein diet could be shown to stimulate the R.E.S. We have no evidence that this is so but the hypothesis could easily be tested and, if confirmed, might be of some practical value. It seems possible that the effect of high protein diet upon the R.E.S. might result from the elevated basal metabolism attributable to high protein diets. However, preliminary experiments did not show that mice treated with thyroxine were any more susceptible to the lethal effect of a small dose of ectromelia than normal mice. Mackay (personal communication) has shown that mice kept in a cold environment are markedly more susceptible to the lethal effect of ectromelia. However, he found the survival time under these conditions to be shortened with very high doses of virus as well as low doses, so that the effect would seem to be basically different from that which we have studied.

SUMMARY

Inoculation of bacterial endotoxin or saccharated iron oxide (Ferrivenin) increases the survival time of weanling mice (TO strain) inoculated with $10^6 LD_{50}$ ectromelia but such treatment decreases the survival time when they are infected with smaller doses of virus (10^3 — 1 LD_{50}).

The mortality of mice inoculated with endotoxin prior to inoculation with about 1 LD_{50} of virus is 95 per cent compared with 55 per cent for comparable normal mice. The time of inoculation of endotoxin in relation to infection time is not critical: mortality is equally increased compared with controls when endotoxin is inoculated 2 days before or 2 days after infection with 1 LD_{50} of virus.

The pathogenicity of ectromelia is somewhat greater for normal weanling mice of one strain (VS) than for such mice of another strain (TO) but in VS mice the pathogenicity of the virus is very little increased by the inoculation of endotoxin. Consequently, the virus has approximately the same pathogenicity for weanling TO mice treated with endotoxin as for weanling VS mice whether treated with endotoxin or not.

An explanation is offered for the decreased susceptibility to very large doses of ectromelia and the increased susceptibility to smaller doses in mice treated with endotoxin or saccharated iron oxide.

I gratefully acknowledge the gift of endotoxin of *Chromobacterium prodigiosum* from Dr. J. H. Humphrey of the National Institute for Medical Research, the help of Mrs. S. Brownstone in respect of certain experiments with thyroxine and the helpful advice of Dr. D. Rowley and his colleagues at the Wright Fleming

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SURVEY OF PAPERS

TURK has demonstrated the immune-adherence phenomena with normal guinea-pig, rabbit and human sera against various bacteria and starch. The non-specific activity is in the euglobulin and is absorbed by many polysaccharides. Much of the specific activity is in the pseudoglobulin and appears to be antibody (p. 97).

WEINBREN and FITSCHEN have inoculated rats with sublethal doses of sodium fluoroacetate at various intervals following partial hepatectomy. They found that hepatic mitoses were decreased when the drug was given 8 hr. after operation, but not when given 24 hr. Repeated doses resulted in an increase in mitoses after 4 days (p. 107).

WARDLAW and HOWARD perfused rat livers with suspensions of bacteria in Ringer-Locke, heated serum or unheated serum, and measured the resulting loss of bacteria from the suspensions. Some Gram-positive and flagellate Gram-negative strains were phagocytosed with unexpected efficiency from Ringer-Locke (p. 113).

NOSSAL has continued his study of single cell antibody production. Here he has shown that, in the primary response, only 2·3 per cent of single cells from draining lymphatic glands formed detectable antibody. In the secondary response 14 per cent of single cells formed antibody and the average amount formed was considerably in excess of that formed in the primary response (p. 118).

WIDDICOMBE, HUGHES and MAY infected the lungs of rabbits by exposing the animals to a cloud of singly dispersed *Brucella suis* organisms. Then, while intra-oesophageal and intratracheal pressures were recorded, they inflated the lungs with measured volumes of air impelled at various known speeds. They found that infection decreased the rigidity of the lung at inflation volumes above the eupnoeic range, and increased the rate of formation of oedema fluid in perfused lungs (p. 125).

LIU, CARTER, SANDERS, SMITH and BETTYLEE HAMPIL injected monkeys intraspinally and intracerebrally with Type I (Mahoney) Poliovirus, and then treated them with hyperimmune rabbit serum. Mortality and spread of paralysis were decreased in both cases.
They found γ-globulin prepared from the serum of human donors immunized with Salk vaccine was similarly efficacious in the treatment of intraspinally injected monkeys (p. 133).

BRUMFITT and HEPTINSTALL introduced suspensions of *Bact. coli* into the jugular veins of rats in which the left renal vein had been partially blocked by a silver clip. After 7 days most of the affected left kidneys showed higher bacterial counts than did controls. This increased count was not found in the first hour after injection (p. 145).

LIKAR and WILSON have investigated further the factors which influence the yield of poliovirus in various tissue and cell cultures. Treatment of the cultures with insulin for 66 hr. or more before infection increased the production of virus. This effect was evident only with incubation below 39·5°, which temperature is by itself effective in enhancing the virus yield (p. 149).

NAIRN, FRASER and CHADWICK have determined the site of production of the pressor enzyme, renin, in the kidney. An antirenin serum was prepared by immunizing rabbits with a purified extract of pig renin. Frozen sections of the pig kidney, fixed in 95 per cent ethanol, were treated with the antiserum which becomes attached to the corresponding antigen in the tissue ; the antiserum was itself traced with a potent goat antirabbit globulin

labelled with lissamine rhodamine B 200, and the sections were examined by fluorescence microscopy. The fluorescent staining was glomerular in distribution and it appears that renin is located in the epithelial cells of the glomeruli (p. 155).

CHEN, CHOU and LIU pre-treated guinea-pigs for 20 days with various antigens, then sensitized them with bovine serum. After 20 more days the animals were challenged with bovine serum intravenously and the incidence of anaphylaxis recorded. The pre-treating antigens were ranged in the following order of efficacy in interfering with anaphylaxis: tetanus toxoid, sheep red cells, diphtheria toxoid and killed *Salm. typhi*. Pertussis vaccine, though most potent in stimulating γ-globulin production, did not interfere with anaphylaxis (p. 164).

BRAS, GOLDBLATT and GYÖRGY fed many hundreds of rats on protein deficient diets to produce experimental cirrhosis. The livers showed fatty change, cellular infiltration, evidence of liver cell degeneration and necrosis with eventual fibrosis. The authors conclude that the cellular infiltration was induced by the necrosis of the liver cells and that the ensuing fibrosis was predominantly due to the preceding necrosis (p. 172).

WILLIAMS describes the histological changes produced in the subcutaneous and intradermal tissues as a result of injection of carragenin into scorbutic guinea-pigs.

The author finds that vitamin C arrests normal fibroblastic development, interferes with the synthesis of collagen and alters the normal pattern of mucopolysaccharide production. In addition the dermal collagen of the scorbutic animal is less susceptible to breakdown than that of normal animals. It is concluded that both connective tissue fibre synthesis and breakdown are inter-related since both are inhibited in the absence of vitamin C (p. 176).

GIBSON produced partial obstruction of the inferior vena cava above the liver of the rat, using a trans-thoracic approach. In the 55 surviving animals the operation was followed by splenomegaly, hepatic congestion and a slowly progressive form of liver disease with focal necrosis culminating in liver fibrosis with liver cell regeneration. These changes are compared with lesions described in other animals and their relation to human disease is discussed (p. 183).

GLEDHILL has studied the effects in mice of injections of bacterial endotoxin or of saccharated iron oxide on subsequent infection with ectromelia virus. With very large infective doses the survival time was increased but with smaller doses one of the two strains of mice used showed a significant decrease in survival time (p. 195).